The ecology of
Hendra virus and Australian bat lyssavirus

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Declaration of Originality

The work presented in this thesis is, to the best of my knowledge and belief, original, and my own work except as acknowledged. The material has not been submitted, either whole or in part, for any other degree at this or any other university.

Hume Field
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Abstract

Chapter one introduces the concept of disease emergence and factors associated with emergence. The role of wildlife as reservoirs of emerging diseases and specifically the history of bats as reservoirs of zoonotic diseases is previewed. Finally, the aims and structure of the thesis are outlined.

In Chapter two, the literature relating to the emergence of Hendra virus, Nipah virus, and Australian bat lyssavirus, the biology of flying foxes, methodologies for investigating wildlife reservoirs of disease, and the modelling of disease in wildlife populations is reviewed.

Chapter three describes the search for the origin of Hendra virus and investigations of the ecology of the virus. In a preliminary survey of wildlife, feral and pest species, 6/21 *Pteropus alecto* and 5/6 *P. conspicillatus* had neutralizing antibodies to Hendra virus. A subsequent survey found 548/1172 convenience-sampled flying foxes were seropositive. Analysis using logistic regression identified species, age, sample method, sample location and sample year, and the interaction terms age*species and age* sample method as significantly associated with HeV serostatus. Analysis of a subset of the data also identified a significant or near-significant association between time of year of sampling and HeV serostatus. In a retrospective survey, 16/68 flying fox sera collected between 1982 and 1984 were seropositive. Targeted surveillance of non-flying fox wildlife species found no evidence of Hendra virus. The findings indicate that flying foxes are a likely reservoir host of Hendra virus, and that the relationship between host and virus is mature.

The transmission and maintenance of Hendra virus in a captive flying fox population is investigated in Chapter four. In study 1, neutralizing antibodies to HeV were found in 9/55 *P. poliocephalus* and 4/13 *P. alecto*. Titres ranged from 1:5 to 1:160, with a median of 1:10. In study 2, blood and throat and urogenital swabs from 17 flying foxes from study 1 were collected weekly for 14 weeks. Virus was isolated from the blood of a single aged non-pregnant female on one occasion. In study 3, a convenience sample of 19 seropositive and 35 seronegative flying foxes was serologically monitored monthly for all or part of a two-year period. Three individuals (all pups born during the study) seroconverted, and three individuals that were seropositive on entry became seronegative. Two of the latter were pups born during the study period. Dam serostatus and pup serostatus at second bleed were strongly associated when data from both years

were combined (*p*<0.001; RR=9, 95%CI 1.42 to 57.12). The serial titres of 19 flying foxes monitored for 12 months or longer showed a rising and falling pattern (10), a static pattern (1) or a falling pattern (8). The findings suggest latency and vertical transmission are features of HeV infection in flying foxes.

Chapter five describes Australian bat lyssavirus surveillance in flying foxes, insectivorous bats and archived museum bat specimens. In a survey of 1477 flying foxes, 69/1477 were antigen-positive (all opportunistic specimens) and 12/280 were antibody-positive. Species (*p*<0.001), age (*p*=0.02), sample method (*p*<0.001) and sample location (*p*<0.001) were significantly associated with fluorescent antibody status. There was also a significant association between rapid focus fluorescent inhibition test status and species (*p=0.01*), sample method (*p=0.002*) and sample location (*p*=0.002). There was a near-significant association (*p*=0.067) between time of year of sampling and fluorescent antibody status. When the analysis was repeated on *P. scapulatus* alone, the association stronger (*p*=0.054). A total of 1234 insectivorous bats were surveyed, with 5/1162 antigen–positive (all opportunistic specimens) and 10/390 antibody-positive. A total of 137 archived bats from 10 species were tested for evidence of Australian bat lyssavirus infection by immunohistochemistry (66) or rapid focus fluorescent inhibition test (71). None was positive by either test but 2 (both *S. flaviventris*) showed round basophilic structures consistent with Negri bodies on histological examination. The findings indicate that Australian bat lyssavirus infection is endemic in Australian bats, that submitted sick and injured bats (opportunistic specimens) pose an increased public health risk, and that Australian bat lyssavirus infection may have been present in Australian bats 15 years prior to its first description.

In Chapter six, deterministic state-transition models are developed to examine the dynamics of HeV infection in a hypothetical flying fox population. Model 1 outputs demonstrated that the rate of transmission and the rate of recovery are the key parameters determining the rate of spread of infection, and that population size is positively associated with outbreak size and duration. The Model 2 outputs indicated that that long-term maintenance of infection is inconsistent with lifelong immunity following infection and recovery.

Chapter seven discusses alternative hypotheses on the emergence and maintenance of Hendra virus and Australian bat lyssavirus in Australia. The preferred hypothesis is that both Hendra virus and Australian bat lyssavirus are primarily maintained in *P. scapulatus* populations, and that change in the population dynamics of this species due to ecological changes has precipitated emergence.

Future research recommendations include further observational, experimental and/or modeling studies to establish or clarify the route of HeV excretion and the mode of transmission in flying foxes, the roles of vertical transmission and latency in the transmission and maintenance of Hendra virus in flying foxes, and the dynamics of Hendra virus infection in flying foxes.

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List of Abbreviations

AAHL	Australian Animal Health Laboratory, Geelong, Victoria, Australia
ABLV	Australian bat lyssavirus
AIDS	acquired immune deficiency syndrome
ARI	Animal Research Institute, Department of Primary Industries and Fisheries, 665 Fairfield Road, Yeerongpilly, Brisbane, Queensland, Australia
AQIS	Australian Quarantine Inspection Strategy
BSL4	Biosafety Level 4
BSE	bovine spongiform encephalopathy
CDC	Centers for Disease Control and Prevention
CI	confidence interval
CMR	Capture-mark-recapture
CNS	Central nervous system
CPE	Cytopathic effect
CRV	Classical rabies virus
CSIRO	Commonwealth Scientific and Industrial Research Organisation
CSF	Cerebrospinal fluid
DAFF	Department of Agriculture, Forestry and Fisheries, Canberra
DPI&F	Department of Primary Industries and Fisheries, Queensland (known prior to May 2004 as the Department of Primary Industries)
EBL	European bat lyssavirus
ELISA	enzyme linked immunosorbent assay
EM	Electron microscopy
EMV	Equine morbillivirus
FAT	fluorescent antibody test
GIS	Geographic information system
HeV	Hendra virus
HIV	human immunodeficiency virus
HPAI	highly pathogenic avian influenza
HRP	horse radish peroxidase
ICTV	International Congress on the Nomenclature of Viruses
IHC	immunohistochemistry
IU	International units
JEV	Japanese encephalitis virus
LPMV	La Piedad Michoacan virus
$MICED_{50}$	mouse intracerebral mean effective dose, 50% lethal dose
NATA	National Association of Testing Authorities

NiV	Nipah virus
NSW	New South Wales
NT	Northern Territory
ONARR	Orphan Native Animal Rear and Release Inc., a volunteer wildlife carer group in southeast Queensland
OR	odds ratio
PC3	Physical Containment Level 3, as defined in the Australia/New Zealand Standard AS/NZS 2243.3:1995
PC4	Physical Containment Level 4, as defined in the Australia/New Zealand Standard AS/NZS 2243.3:1995
PCR	polymerase chain reaction
pers. comm.	personal communication
PI	post-inoculation
Qld	Queensland
QM	Queensland museum
RFFIT	rapid focus fluorescent inhibition test
RK	rabbit kidney
ROC	receiver-operating characteristic
RR	relative risk
SARS	sudden acute respiratory syndrome
Se	sensitivity
SIR	susceptible-infectious-removed
SNT	Serum neutralization test
Sp	specificity
TCID	tissue culture infective dose
UK	United Kingdom
UQ	The University of Queensland, Brisbane, Australia
USA	United States of America
vCJD	variant Creutsfeld-Jacob disease
YVL	Yeerongpilly Veterinary Laboratory, Animal Research Institute, Department of Primary Industries and Fisheries, 665 Fairfield Road, Yeerongpilly, Brisbane, Queensland, Australia.
WA	Western Australia

CHAPTER ONE

INTRODUCTION

While the current emphasis on emerging diseases in the scientific literature and in the popular press suggests otherwise, novel diseases have occurred throughout history. By definition, every newly identified disease is novel. Today's endemic disease is figuratively yesterday's novel disease. This observation is not meant to invoke a complacency regarding the inevitability of disease emergence, nor to downplay the need for surveillance, nor to discount the challenges associated with investigation and managing outbreak of new diseases. Rather, it offers a window to the lessons of history. The outcome of investigations of cholera epidemics by Dr. John Snow in London in the 1880s illustrated for the first time how the actions of man could precipitate the emergence of disease. At the same time, it also demonstrated the value of an epidemiological approach in the investigation of a disease outbreak. Observing the pattern of disease, Snow hypothesized that a faeces-contaminated water supply was the primary mode of transmission of the diarrhoea-causing cholera. Analysing data from the Bills of Mortality, he was able to demonstrate a close association between frequency and occurrence of cholera, and certain water-supply companies. It was subsequently shown that the companies in question drew their water downstream from the sewage outlet in the Thames River (Martin *et al.*, 1988). The research presented in this thesis will apply the same epidemiological approach to the investigation of two recently emerged infectious disease of public and animal health significance in Australia.

1.1 Disease emergence and factors associated with emergence

AIDS is the most familiar and significant emergent disease threatening public health in the world today. The emergence and spread of the causal human immunodeficiency virus highlights many of the issues frequently associated with emerging diseases in general: the sudden appearance and cryptic origins; limited prophylactic and therapeutic drugs; and social, cultural, demographic and technological risk factors. SARS, the haemorrhagic fevers (Ebola, Hantaan, Crimea-Congo), Nipah virus encephalitis, hepatitis C and E, variant Creutsfeld-Jacob disease, and most recently, highly pathogenic avian influenza are other examples of emerging infectious diseases threatening public health. But emerging

diseases are not confined to human populations. The novel prion disease BSE, first identified in dairy cattle in the UK in 1986 (Wilesmith *et al.*, 1988), has had dramatic repercussions on animal husbandry and trade in the UK and Europe, and more recently Japan, Canada and the USA. The putative association between consumption of BSE-infected products and vCJD in humans has added a public health dimension of unknown proportions. Disease emergence has also impacted wildlife populations. Phocine distemper, first recognized in harbour seals (*Phoca vitulina*) in Europe in 1988 resulted in the death of more than 17,000 seals from Denmark to the North Sea, the Wadden Sea and the Baltic Sea (Osterhaus *et al.*, 1990). A chronology of selected recently emerged viral diseases is presented in Table 1.1.

Table 1.1: Examples of infectious viral agents and disease that have emerged since 1973[1].

Year	Viral agent	Disease syndrome in humans
1973	Rotavirus	Infantile diarrhoea
1974	Barmah Forest virus	Polyarthritis
1977	Ebola virus	Haemorrhagic fever
1977	Hantaan virus	Haemorrhagic fever with renal syndrome
1983	Human immunodeficiency virus	Acquired immunodeficiency syndrome
1988	Hepatitis E	Enterically transmitted hepatitis
1988	Human herpesvirus-6	Roseola subitum
1989	Hepatitis C	Parentally transmitted hepatitis
1991	Guanarito virus	Venezuelan haemorrhagic fever
1993	Hantavirus	Hantavirus pulmonary syndrome
1994	Sabia virus	Brazilian haemorrhagic fever
1994	Hendra virus	Respiratory and neurological disease
1996	Australian bat lyssavirus	Neurological disease
1997	Menangle virus	Febrile disease
1999	Nipah virus	Neurological disease
2003	SARS coronavirus	Sudden Acute Respiratory Syndrome
2004	Highly pathogenic avian influenza	Respiratory disease

[1] Adapted from Longbottom (1997) and Satcher (1995).

What constitutes an emerging disease? Definitions encompass not only those diseases associated with novel agents, but also those known diseases that are rapidly increasing in incidence and/or distribution. The term 're-emerging' is sometimes applied to the latter (Morse, 1995). Thus, SARS, identified in 2003, and Japanese encephalitis virus, long-recognized elsewhere but currently emerging in northern Australia, could both be

regarded as emerging diseases. But are so-called emerging diseases really emerging, or are they an artifact of improved diagnostic capabilities and enhanced disease surveillance activities? Satcher (1995) for example, discusses the role of nucleic acid amplifying techniques for detecting and identifying otherwise non-cultivable agents. While there is little doubt that these factors contribute to our ability to accurately describe the occurrence and frequency of known disease, improved surveillance efforts or diagnostic capabilities cannot adequately explain the emergence *per se* of disease. So what triggers disease emergence? Modern epidemiological principles contend that disease is multi-factorial: that, in addition to the presence of the infectious agent, additional factors are generally necessary for infection and disease to occur. Such factors may relate to the agent, to the host, or to the environment. Morse (1995) contends that factors that precipitate disease emergence can frequently be identified, either specifically or broadly. He categorizes putative causal factors for emergence as follows:

- Ecological changes (including those resulting from agricultural development);
- Changes in human demographics and behaviour;
- Increased international travel and commerce;
- Advances in technology and industry;
- Microbial adaptation or change;
- Breakdown of public health measures.

Within this framework, Table 1.2 provides several examples of disease emergence and the putative factors associated with their emergence. Lederberg *et al* (1992) describe these changes as providing an ‘epidemiological bridge’ that facilitates contact between the agent and naive population.

Table 1.2: Putative factors in disease emergence[1]

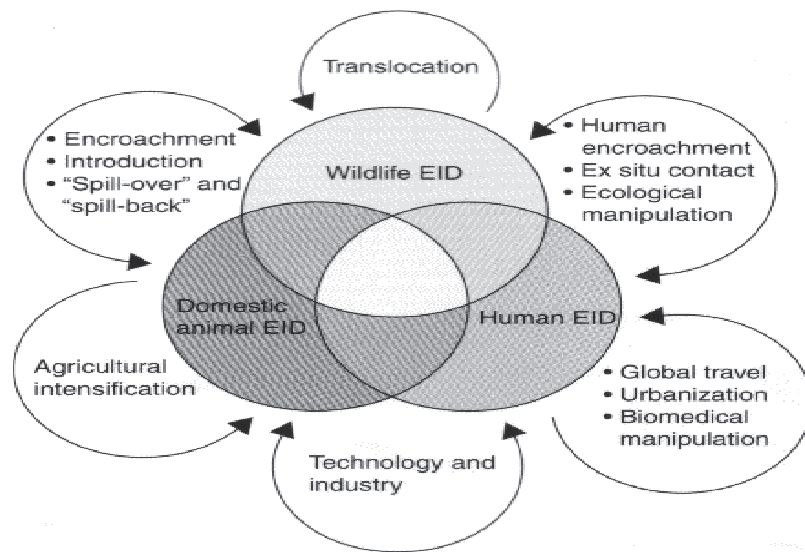
Underlying factor	Example of factor	Example of disease
Ecological changes	Changes in water ecosystems. Land-use changes. Climatic changes.	Schistosomiasis, Rift Valley fever, hantavirus pulmonary syndrome.
Human demographic, societal or behavioural changes	Population growth and movement. High-density habitation. Human conflicts. Intravenous drug use. Sexual behaviour.	AIDS, SARS, hepatitis C, ebola haemorrhagic fever.
Ease, extent and frequency of international travel	Worldwide movement of goods and people.	'Airport' malaria, dissemination of mosquito vectors, rat-borne hantaviruses.
Technology and industry	Changes in food processing and packaging. Globalisation of food industries. Increased frequency of medical/surgical transplants. Increased use of immunosuppressant drugs.	BSE, E. coli haemolytic uraemic syndrome, SARS, transfusion-associated AIDS and hepatitis, opportunistic infections in immunosuppressed patients.
Microbial adaptation and change	Microbial evolution. Response to environmental selection pressures.	'Antigenic drift' in influenza viruses, multiple antibiotic resistant bacterial diseases.
Inadequate public health measures	Inadequate water quality, sanitation, and vector control. Reduction of disease prevention programs.	Tuberculosis resurgence in USA, diptheria resurgence in former USSR, cholera in refugee camps in Africa.

[1] Adapted from Longbottom (1997) and Morse (1995).

1.2 Wildlife as reservoirs of emerging diseases

Daszak *et al* (2000) regard disease emergence as primarily an ecological process, with emergence frequently resulting from a change in the ecology of the host or the agent or both. They argue that most emerging diseases exist within a finely balanced host-agent continuum between wildlife, domestic animal and human populations (Figure 1.1). Morse (1995) also contends that wildlife play a key role in the emergence of zoonotic disease by providing a 'zoonotic pool' from which previously unknown pathogens may emerge.

Figure 1.1: The host-agent ecological continuum[1]



[1] From Daszak *et al* (2000).

Certainly there is an increasing body of evidence attributing many of the novel public health diseases to zoonotic origins (Fiennes, 1978; McNeill, 1976). In a comprehensive review seeking to identify risk factors for disease emergence, Taylor *et al* (2000; 2001) concluded that emerging diseases were three times more likely to be associated with zoonotic pathogens than with non-zoonotic pathogens. In addition, infectious agents of wildlife represent an increasing proportion of emerging infectious diseases, a scenario consistent with an increasing human exposure to wildlife species or habitats. This increased exposure is considered to be primarily a result of land-use changes and demographic shifts. For example, in 1993 a previously unknown group of Hantaviruses emerged in the USA, responsible for an acute respiratory syndrome (with a case fatality rate of 50%) in humans. Subsequent investigations indicated that Hantaviruses had long existed in rodent populations in the USA, but that recent ecological changes favouring increased rodent populations promoted increased human exposure opportunities (Schmaljohn & Hjelle, 1997). The emergence of Nipah virus from fruit bats in northern peninsular Malaysia in 1999 is another example. It has been suggested (Field *et al.*, 2001) that the emergence of Nipah virus was (in part) associated with the encroachment of commercial pig farms into forested areas of high fruit bat activity. Once the virus 'spilled over' into the immunologically naïve pigs, high pig and farm densities then facilitated the

rapid dissemination of the infection within the local pig population. The movement of pigs for sale and slaughter in turn led to the rapid spread of infection to southern peninsular Malaysia and Singapore, where the high-density, largely urban pig populations facilitated transmission of the virus to humans.

1.3 Bats (*Chiroptera*) as reservoirs of zoonotic disease

The behavioural ecology of many bat species identifies them as potentially efficient vertebrate disseminator hosts of mammalian viruses. In addition, it has been suggested that bats are unique in their response to viral infections, in that they are able to sustain viral infections in the absence of overt disease (Sulkin & Allen, 1974). Historically, a wide range of viral infections (including flaviviruses, alphaviruses, rhabdoviruses, arenaviruses, reoviruses, and paramyxoviruses) has been identified in bats (Sulkin & Allen, 1974). More recently, a number of emerging zoonotic diseases of viral origin have been linked to bats (Hoar *et al.*, 1998). These include Hantaan virus, isolated from an insectivorous bat in Korea; Rift Valley fever virus, isolated from bats in the Republic of Guinea; a strain of yellow fever isolated from a fruit bat in Ethiopia; and serologic evidence of Venezuelan equine encephalitis, St Louis encephalitis, and eastern equine encephalitis viruses in Guatemalan bats. In experimental inoculations of plants and animals to identify potential natural hosts of Ebola virus, high titres of virus were recovered from the pooled sera and viscera of fruit bats, and virus was recovered from the faeces of one bat 21 days post-inoculation (Hoar *et al.*, 1998). While bat-variant rabies has long been recognized in the USA, the incidence of human rabies cases attributed to that variant has increased in recent years (McColl *et al.*, 2000).

However the role of bats in the epidemiology of many of these diseases is unclear. This uncertainty can be (at least in part) due to difficulties associated with disease investigations of wildlife populations. Many of these challenges are the result of the uncontrolled nature of wildlife populations, where basic parameters such as population size, age and sex structure, and rates of immigration, emigration, birth and mortality are frequently unknown. Additional constraints can be posed by a poorly understood species biology, invalid or inadequate diagnostic tests, statutory protection afforded by the species conservation status, and public opposition by individuals or groups.

1.4 The thesis – aims and structure

Against this background of disease emergence, of the increasing zoonotic nature of emerging agents, of wildlife species as reservoirs of novel diseases, and of challenges posed by wildlife disease studies, I present a research program which investigated the ecology of two recently emerged and zoonotic agents in bats in Australia: Hendra virus and Australian bat lyssavirus. The emergence of these two agents had significant public and animal health impacts, and fostered a heightened awareness of emerging infections disease in Australia. This chapter has briefly introduced infectious disease emergence and associated concepts. Chapter 2 presents a comprehensive literature review of the emergence and description of both agents, of the biology of fruit bats of the genus *Pteropus* (commonly known as flying foxes) in Australia, and of methodologies for investigating wildlife reservoirs of disease. My original research encompasses the preliminary surveillance of wildlife populations for evidence of Hendra virus infection (Chapter 3), the subsequent targeted surveillance of flying fox populations to elucidate the ecology of Hendra virus in both free-living (Chapter 3) and captive (Chapter 4) populations, a cross sectional study series to describe the ecology of ABLV in wild bat populations (Chapter 5), a deterministic state-transition model of Hendra virus infection in a hypothetical flying fox population (Chapter 6), and a general discussion chapter (Chapter 7).

The impact and consequences of the emergence of these two agents has highlighted not only the threat posed by emerging diseases to animal and public health, but the need for effective surveillance strategies for wildlife populations. A solid understanding of the ecology of the agents and the natural hosts are an integral part of such a strategy.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

The sudden emergence of Hendra virus and Australian bat lyssavirus in Australia, and Nipah virus in Malaysia poses a number of important questions on the ecology and pathogenesis of each. What are their natural hosts? Why did they emerge at this time? What factors precipitated their emergence? What are the spillover mechanisms? What is the potential impact on domestic species? This chapter encompasses a comprehensive review of the current state of knowledge of the emergence, phylogeny, natural and experimental infections of Hendra virus and Australian bat lyssavirus, and in doing so, addresses some of these questions while posing others for further investigation. Because of the numerous parallels between Hendra virus and Nipah virus, a review of the latter is also included.

2.2 HENDRA VIRUS

2.2.1 The emergence of Hendra virus

Hendra virus was first described in 1994 in Australia after a sudden outbreak of an acute respiratory syndrome in thoroughbred horses in a Brisbane training stable (Murray *et al.*, 1995b). The syndrome was characterized by severe respiratory signs and high mortality. The causal agent was initially unknown. Exotic diseases, including African horse sickness, and a number of toxic agents were excluded (Douglas *et al.*, 1997; Douglas, 1998). A previously undescribed virus of the family *Paramyxoviridae* was subsequently identified as the causal agent. The virus was initially named equine morbillivirus, but was later re-named Hendra virus (after the Brisbane suburb where the outbreak occurred). There have been three known foci of natural Hendra virus infection in horses: the first occurred near Mackay in August 1994, the second in Brisbane in September 1994, and the third in Cairns in January 1999. While chronologically preceding the Brisbane outbreak, the Mackay incident was not identified until October 1995.

2.2.2 Natural infections in horses and humans

The putative index case in the Brisbane outbreak was a heavily pregnant thoroughbred mare named Drama Series, at pasture in the Brisbane suburb of Cannon Hill. When observed to be ill (September 7, 1994), she was moved to a training stable housing 23 other thoroughbreds in the Brisbane suburb of Hendra, where she died after a 2-day illness. A further twelve horses[1] in the stable and an adjoining training stable died acutely over the subsequent fourteen days (Figure 2.1) (Murray *et al.*, 1995a). Clinical signs included fever, facial swelling, severe respiratory distress, ataxia, and terminally, copious frothy (sometimes blood-tinged) nasal discharge. There were four non-fatal cases, two of which retained mild neurological signs. A further three horses in the stable were subsequently found to have seroconverted without demonstrable clinical signs. All seven were subsequently euthanased (Baldock *et al.*, 1996; Douglas *et al.*, 1997).

Figure 2.1: Chronology of equine and human cases of disease attributed to Hendra virus infection[1]

	September 1994							
	7	9	13	14	15	16	17	19-26
Horses								
Cannon Hill (Paddock)	2 horses moved							
Hendra (Stables)		Mare died				2 horses moved		10 horses dead 4 recovered
Hendra (Neighboring property)		1 horse moved						1 horse dead 1 recovered
Kenilworth (150 km distant)								1 horse dead 1 recovered
Samford (Paddock)								1 recovered
			New South Wales					
Humans								
Stablehand				Becomes ill				Slow recovery
Trainer					Becomes ill		Hospitalized	Died

[1] From Murray *et al* (1995a)

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[1] Some authors report the number of fatal equine cases as 14. This figure includes an earlier horse fatality (of unknown aetiology) in the index case paddock that was initially counted as a case when preliminary investigations (wrongly) indicated that the date of its death preceded the onset of illness in the putative index case by less than the estimated incubation period of the disease. Subsequent inquiries revealed that the horse had died more than a month earlier (Ian Douglas, DPI&F, pers. comm.)

The trainer and a stable hand, both of whom were directly involved in nursing the index case, became ill with a severe influenza-like illness within a week of contacting the index case. The trainer was hospitalised and subsequently died after respiratory and renal failure. Infection with Hendra virus was demonstrated in both cases (Selvey *et al.*, 1995).

In October 1995, an outbreak near Mackay in central Queensland (800 kilometres north of Brisbane) was retrospectively diagnosed after the Hendra virus-attributed death of a stud-owner who suffered a relapsing encephalitic disease. Two horses were infected, both fatally (Hooper *et al.*, 1996; Rogers *et al.*, 1996). The first horse, a 10-year-old heavily pregnant thoroughbred mare died on August 1, 1994 after exhibiting severe respiratory distress, ataxia, and marked swelling of the cheeks and supraorbital fossa over a 24-hour period. The second horse, a two-year-old colt in an adjoining paddock was reported to have licked the muzzle of the dead mare. The colt died 11 days later, again after a 24-hour clinical course, during which he exhibited aimless pacing, muscle trembling and haemorrhagic nasal discharge. Histopathology examinations at the time were inconclusive in both cases. Avocado poisoning and brown snakebite were considered as differential diagnoses (Allworth *et al.*, 1995).

Serological studies were an integral part of the outbreak investigations of the Brisbane and Mackay incidents. No evidence of Hendra virus infection was found in 800 domestic animals surveyed on the case properties or on in-contact properties. They included 387 horses, 287 cattle, goats and pigs, 23 dogs, 64 cats, and 39 poultry (Baldock *et al.*, 1996; Rogers *et al.*, 1996). Particular effort was directed towards surveying the broader Queensland horse population, with a further 2024 horses from 166 properties surveyed in a structured survey (Ward *et al.*, 1996). With the exception of the 7 horses that survived infection in the Hendra outbreak, none of the surveyed domestic animals showed serological evidence of exposure to Hendra virus. The negative findings of the highly sensitive ‘gold standard’ serum neutralisation test provided a level of confidence that Hendra virus was not established in the Queensland horse population, and that in-contact domestic animals were not the source of infection. Because of the temporal proximity of the Mackay and Brisbane incidents, efforts were made to identify possible links between the two properties. These investigations, undertaken in late 1995, and focused primarily on horse movements, personnel movements, and management practices, found no evidence to directly link the two outbreaks. However, a number of common features were noted (Baldock *et al.*, 1996):

- o The temporal period - August/September 1994;
- o The putative index case - an older heavily pregnant mare at pasture;
- o The length of time the putative index case had been in the paddock – well in excess of the incubation period of the disease observed in other horses;
- o Absence of infection in other horses in the same paddock;
- o Transmission only from the index case;
- o Infection transmitted from horse to human.

Baldock *et al* (1996) also contended that the pattern of the Brisbane outbreak suggested that Hendra virus infection was not highly contagious in horses, and probably required direct contact or mechanical transmission of infectious body fluids for natural transmission to occur. Subsequent experimental trials supported these field observations (see Section 2.2.4).

In January 1999, four and a half years after the previous cases, the third focus of Hendra virus infection was reported in a horse near Cairns in north Queensland. The affected horse was again an aged thoroughbred mare at pasture. Clinical signs included inappetence, depression and swelling of the face, lips and neck. Despite symptomatic treatment, the mare deteriorated and was found recumbent the next morning with copious quantities of yellow frothy nasal discharge, and was euthanased. A companion horse was unaffected on clinical and serological examination (Field *et al.*, 2000). A number of epidemiological features of this case were also common to the Mackay and Brisbane incidents. On all three occasions, the index case was in the paddock for longer than the incubation period calculated from field observations (8-16 days) (Baldock *et al.*, 1996) and in experimental infections (4-10 days) (Murray *et al.*, 1995b). From this, we can reasonably conclude that the index case on each occasion was exposed and infected in the paddock within two weeks of the onset of clinical disease. On each of the three occasions, breed (Thoroughbred), sex (female), age (>8yo) and housing (paddocked) have been consistent putative horse-level risk factors for the index case. Reproductive status may be an additional risk factor: both the Mackay and Brisbane index cases were heavily pregnant. The Cairns case should have been in mid-pregnancy according to service dates and pregnancy diagnosis, but was not pregnant on necropsy (Field *et al.*, 2000).

Flying foxes have been demonstrated to be a natural host of Hendra virus (see Chapter three). The presence of favoured flying-fox food trees in the paddock of index cases is a consistent paddock-level risk factor. Further, in at least the Brisbane and Mackay

outbreaks, disease in the index case coincided with late gestation in the (seasonally breeding) flying foxes in those areas (Field *et al.*, 2000). The possible significance of these observations is examined in later chapters.

2.2.3 Ultrastructural, molecular and phylogenetic studies of Hendra virus

Ultrastructural studies (Hyatt & Selleck, 1996; Murray *et al.*, 1995b) of the newly identified virus showed it to be pleiomorphic, ranging in size from 38 to more than 600nm, and enveloped, with 10-18nm surface projections. Nucleocapsids were 18nm wide and exhibited a herringbone pattern with a 5nm periodicity. These features indicated that the virus was a member of the family *Paramyxoviridae*, possibly genus *Paramyxovirus* or *Morbillivirus*. Antisera from a range of paramyxoviruses, morbilliviruses and pneumoviruses failed to neutralise the virus, although very weak immunofluorescent and protein immunoblot reactions to rinderpest antiserum were recorded (Murray *et al.*, 1995b). The virus did not exhibit detectable haemagglutination or neuraminidase activity. The above features suggested the virus was a morbillivirus (Murray *et al.*, 1995b). Comparative sequence analyses by PCR of a portion of the matrix protein supported this, with phylogenetic analysis indicating that the virus was distantly related to other known morbilliviruses (Murray *et al.*, 1995b). Hence the name equine morbillivirus was tentatively ascribed to the virus. Importantly, it was noted that the phylogenetic analysis suggested that the virus had not resulted from single or multiple point mutations from a closely related virus, and that emergence from a natural host was the most probable explanation of its origin (Murray *et al.*, 1995b).

Subsequent studies of the complete nucleotide sequence of the matrix (M) and fusion (F) proteins, and partial sequence information from the PV proteins, confirmed that a greater homology existed between EMV and known morbilliviruses than between EMV and other genera of the family *Paramyxoviridae* (Gould, 1996). Notwithstanding, sequence comparisons revealed a large degree of divergence with other morbilliviruses, leading the author to note that an argument could be made for placing EMV in a new genus, and that additional sequence data was necessary to determine the precise position of EMV within the family.

Sequencing of the entire genome confirmed EMV as a member of the subfamily *Paramyxovirinae*, but identified differences that supported the creation of a new genus. These differences included a larger genome size, the replacement of a highly conserved

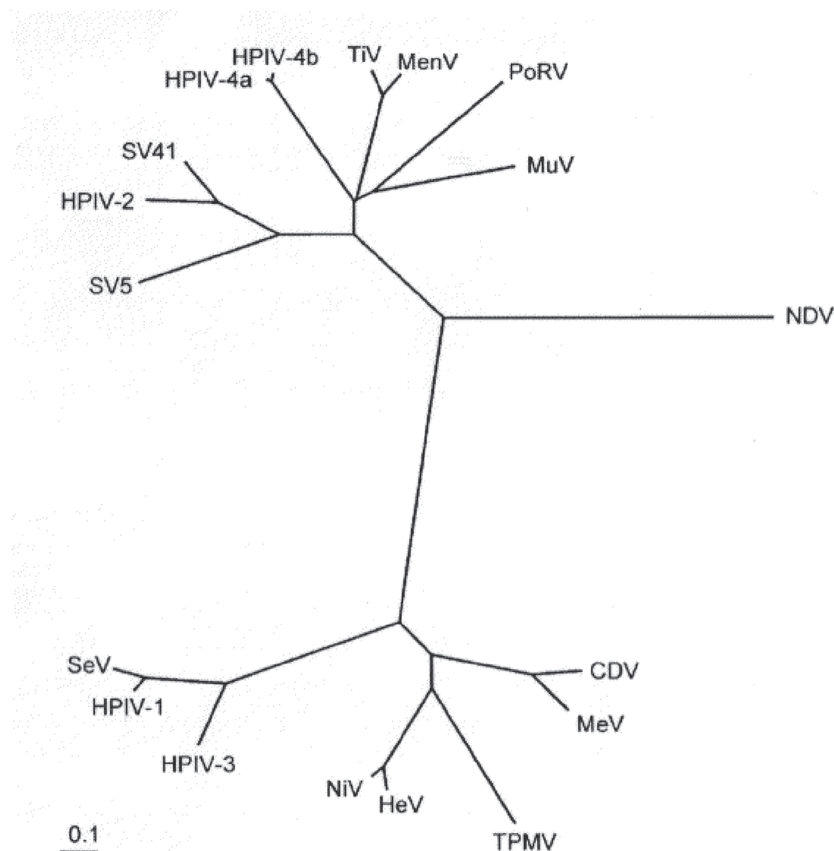
sequence in the L protein gene, different genome end sequences, and other sequence and molecular features (Wang *et al.*, 2000). The authors proposed *Henipavirus* as the new genus, with Hendra virus (see below) the type species and Nipah virus (see Section 2.3.3) the second member. Concurrently, it was being argued that the name 'equine morbillivirus' was inappropriate as mounting evidence suggested that this was neither an equine virus nor a morbillivirus (Wang *et al.*, 1998; Young *et al.*, 1997). Thus the virus was renamed Hendra virus, after the location of the first known outbreak. The ICTV has formally recognized the genus *Henipavirus*, and the name Hendra virus (ICTV, 2000).

Several other previously unknown members of the family *Paramyxoviridae* have been described in recent years. These include Phocine distemper virus and Cetacean morbillivirus (genus *Morbillivirus*), responsible for disease epidemics in marine mammals (Osterhaus *et al.*, 1990; Taubenberger *et al.*, 1996); Menangle virus (genus *Rubulavirus*), which caused severe reproductive disease in a commercial piggery in Australia in 1997 (Philbey *et al.*, 1998); Nipah virus (genus *Henipavirus*), responsible a major epidemic in pigs and humans in Malaysia in 1998 and 1999 (Chua *et al.*, 2000; Nor *et al.*, 2000); Salem virus (unclassified), possibly associated with a disease outbreak in horses in New Hampshire and Massachusetts, USA in 1992 (Renshaw *et al.*, 2000); Tupaia paramyxovirus (unclassified), isolated from an apparently healthy tree shrew (*Tupaia belangeri*) in Thailand (Tidona *et al.*, 1999); Tioman virus (genus *Rubulavirus*) and Pulau virus (unclassified) isolated from flying foxes in Malaysia during attempts to isolate Nipah virus (Chua *et al.*, 2001). Tioman and Menangle are phylogenetically closely related. Tupaia virus and Salem virus both share some sequence homology with Hendra and Nipah, yet have features that preclude their inclusion as henipaviruses or as morbilliviruses. While Palau virus has yet to be fully characterized, it too appears not to fit readily into either genus. Why are we seeing this intriguing pattern of emergence of previously unknown lineages of paramyxoviruses?

There are two reports of isolations of paramyxoviruses from bats prior to the description of Hendra virus in flying foxes in 1996; a sub-type of parainfluenza virus type 2 from the fruit bat species *Rousettus leschenaulti* in India (Pavri *et al.*, 1971), and Mapuera virus from another fruit bat species, *Sturnira lilium*, in Brazil (Henderson *et al.*, 1995). Both of these belong to the genus Rubulavirus, but are unrelated to Menangle and Tioman viruses. However Mapuera virus is closely related to porcine rubulavirus (formerly LPMV), a novel

paramyxovirus that caused serious disease in pigs in Mexico (Moreno-Lopez *et al.*, 1986). Figure 2.2 presents a phylogenetic representation of the family *Paramyxoviridae*.

Figure 2.2: A phylogenetic representation of the family *Paramyxoviridae*[1]



A phylogenetic tree based on the deduced amino acid sequences of the matrix protein of members of the family *Paramyxoviridae*. Branch lengths represent relative evolutionary distances. NDV= Newcastle disease, CDV = canine distemper virus, MeV= measles virus, TPMV= Tupaia Paramyxovirus, HeV=Hendra virus, NiV=Nipah virus, HPIV3=human parainfluenza virus 3, HPIV1=human parainfluenza virus 1, SeV= Sendai virus, SV5=Simian virus 5, HPIV2=human parainfluenza virus 2, SV41=Simian virus 41, HPIV4a=human parainfluenza virus 4a, HPIV4b=human parainfluenza virus 4b, TiV=Tioman virus, MenV=Menangle virus, PoRV= porcine rubulavirus, MuV=mumps virus.

[1] From Chua *et al* (2002).

2.2.4 Experimental infections in animal species

AAHL is Australia's peak animal health laboratory, and houses the only PC4 laboratory in the southern hemisphere. Because of the classification of Hendra virus as a BSL4 agent, laboratory work in Australia involving live virus is restricted to this facility. In the course of the Hendra outbreak, the facility was used to undertake diagnostic investigations that led to the identification of the novel Hendra virus as the causal agent. In addition, experimental animal infections have been conducted in the AAHL PC4 animal rooms. The first of these was undertaken in the course of the outbreak investigation to determine whether an infectious agent was indeed responsible for the disease outbreak. Two horses were inoculated (one intravenously, one by intranasal aerosol) with tissue homogenates from case horses. Both developed a clinical syndrome consistent with that seen in the Hendra horses. A further two horses were then inoculated with the supernatant from cell cultures inoculated with the original tissue homogenates, one intravenously and one by intranasal aerosol, with 5ml and 10ml respectively of $2x10^7$ $TCID_{50}$ per ml. Both of these horses also developed clinical disease. The clinical manifestations in the four horses, and the recovery of virus from all, served to partially fulfill Koch's postulates, and so provided strong evidence of causality (Murray *et al.*, 1995b).

A series of further experimental inoculations of laboratory species followed, the first (Westbury *et al.*, 1995) prompted by observations that Hendra virus was able to grow in a remarkable range of cell lines, including those derived from mammals, birds, reptiles, amphibians, and fish. Of the mice (10), guinea pigs (5), rats (5), chickens (2), rabbits (2), cats (2) and dogs (2) inoculated subcutaneously with an estimated dose of 5000 $TCID_{50}$, both cats and four of the guinea pigs developed disease. The clinical syndrome in the cats was comparable to that seen in experimentally and naturally infected horses. Equivocal antibody titres (attributed to a serological response to the viral inoculum) were seen in three rats and one dog. Substantial neutralising titres (1:2560 and 1:320) were seen in the rabbits. This serologic response in the absence of clinical disease was inconclusively interpreted as indicative of either sub-clinical infection in the rabbits, or of serologic reaction to the inoculum mass. Given the limited or negative serologic response in the other species, the former interpretation seems more plausible.

A follow-up transmission study sought to investigate the suitability of cats as a model species for horses (Westbury *et al.*, 1996). Six cats were inoculated with a dose of 0.1ml of $10^{3.6}$ TCID per 100μl (two orally, two intranasally, and two subcutaneously). All six

inoculated cats and one of four in-contact control cats developed disease. The mode of transmission to the in-contact cat is unknown. Virus was variously isolated from the trachea, lung, pleural fluid, liver, spleen, lymph node, rectum, brain, and urine of affected cats. The authors imply that urine was the most probable source of infection in the in-contact cat, because sneezing and coughing were not part of the clinical syndrome, and because virus was recovered from 4/4 urine samples and 7/7 kidney samples (and only 1/6 rectal samples) of affected cats. It was observed that transmission did not seem to occur readily, as a second in-contact cat, and two further controls immediately adjacent to affected cats did not succumb to infection.

Later, Williamson *et al* (Williamson *et al.*, 1998) undertook a series of transmission studies in horses, cats and flying foxes[2]. A standard dose of inoculum of 50,000 $TCID_{50}$ was used for all animals in this series except for intranasally inoculated horses, which received a dose of 10^6 $TCID_{50}$. In one study, four horses were experimentally infected, and transmission attempted to three in-contact horses and six cats. Of four horses that were inoculated (two subcutaneously and two intranasally), two developed fatal clinical disease, one (intranasally inoculated) developed a non-fatal febrile illness of 5 days duration, and one subcutaneously inoculated horse exhibited no clinical disease. The latter did not seroconvert, and at post-mortem 21 days PI, had no gross lesions. The non-fatal case, also euthanased at day 21, had a HeV neutralising titre of 1:512, had chronic lesions characterized by marked periarterial mononuclear cell infiltration in a range of organs, but did not exhibit any HeV antigen by immunoperoxidase staining. None of the three in-contact horses and none of the six in-contact cats showed clinical or serologic evidence of infection. In a follow-on study, the three in-contact horses were exposed to 12 cats inoculated by the oral route and housed in cages attached to the rails of the horse stalls. Two cats were euthanased on each day post-inoculation, as part of a separate (unpublished) pathogenesis experiment. Clinical disease was observed in the last two remaining cats euthanased on days 7 and 10 PI. Virus was isolated from the lung, kidney, spleen, blood and urine of these and two other cats. One of the in-contact horses (housed adjacent to two of the above cats) developed a febrile illness six days after last cat contact, and had seroconverted (neutralising titre 1:2) and yielded virus from blood, urine, spleen and kidney at post-mortem four days after the initial temperature rise. The

[2] The identification of flying foxes as a natural host of Hendra virus is an outcome of original research presented in Chapter 3. Chronologically, this research preceded and formed the basis of experimental studies in flying foxes described here.

remaining two in-contact horses were seronegative seven days after last cat contact. The final experiment in this series was undertaken after preliminary wildlife surveillance (Chapter 3) had identified flying foxes as a possible reservoir host. The objective was to experimentally infect flying foxes and attempt transmission to horses. Of eleven captive and HeV seronegative grey-headed flying foxes (*Pteropus poliocephalus*), four were inoculated subcutaneously, four were inoculated intranasally/orally, and three remained uninoculated as in-contact controls. The bat cages were positioned such that the two in-contact horses were exposed to the expired air and urine of the inoculated bats. In addition, urine and faeces from the inoculated bats was added to the drinking water of the horses daily. None of the flying foxes developed clinical disease over the 21-day observation. Neutralising antibody titres (1:40 and 1:80) were found in two of the subcutaneously inoculated bats, and three of the intranasally/orally inoculated bats (1:5, 1:5, and 1:10) at day 21. All inoculated bats were positive by ELISA; all of the in-contact control bats were seronegative by ELISA and SNT. No bats exhibited gross lesions at necropsy. Histological lesions and positive immunostaining were evident in the two bats with titres of 1:40 and 1:80. Virus was not isolated from the tissues, urine or faeces of any bat. The two in-contact horses, which had not seroconverted 14 days after the removal of the bats, were inoculated by subcutaneous injection. Both developed clinical disease (5 and 9 days PI). In this series of trials, Williamson *et al* recovered virus more consistently from the urinary tract of horses than from other possible portals of exit. Virus was isolated from the kidney of all six clinically affected horses, and from the urine of four of the six, suggesting to the authors that urine may be an important route of excretion. Virus was also isolated from the saliva of two horses. The isolation of virus from the spleen of the non-fatal case at necropsy, 21 days PI, and in the presence of a high neutralising antibody titre led the authors to contend that HeV can persist beyond initial infection. This finding has important ramifications for HeV control and eradication measures, and supports the decision at the Hendra outbreak to destroy the surviving seropositive horses. These trials corroborated the earlier experimental observations that HeV is not highly infectious under experimental conditions, and are consistent with field observations both at Hendra and Mackay. Whether the apparent low infectivity is a reflection of the innate infectivity of the virus, the instability of the virus outside the host, or ineffective contact, is unclear. The repeated success in subsequently inducing disease in in-contact control horses by inoculation suggests that host characteristics are not of primary importance in the experimental setting.

In the most recent HeV experimental infection study at AAHL, pregnant guinea pigs and flying foxes were inoculated to investigate transplacental infection as a possible mode of transmission (Williamson *et al.*, 2000). A standard dose of 50,000 $TCID_{50}$ from the same stock as used in all the previous experimental studies was used to infect all animals. In the first trial, 18 of 20 guinea pigs at mid-term pregnancy were inoculated subcutaneously. Two remained as uninoculated controls. Two guinea pigs were killed on each of days 2, 4, and 6 post-inoculation respectively, none exhibiting clinical disease. The remainder were killed either at the onset of attributable clinical disease (9) or at the end of the 23 day trial (3 inoculated guinea-pigs and 3 controls). One guinea pig aborted (three fetuses) on day 10. Virus was recovered from 11 of the 18 inoculated guinea pigs. Of these 11, virus was recovered from the uterus and placenta of 10, with titres equal to, or exceeding, those found in other tissues. In addition, immunopositive staining was observed in uterus, placenta, ovary and foetal tissues. Viral titres in pregnant guinea pigs were observed to be generally higher than in non-pregnant guinea pigs inoculated with the same dose and inoculum in previous experiments. Thus the authors concluded that HeV appears to have a predilection for the reproductive tract, at least in guinea pigs in mid-pregnancy. In the second trial, four pregnant *P. poliocephalus* from a captive colony (serologically negative for HeV) were inoculated subcutaneously with the standard dose described above. Two of the four were killed 10 days PI; the remaining two were bled at day 14 and killed at 21 days PI. Four guinea pigs inoculated as inoculum controls were killed at the onset of clinical disease. All four guinea pigs developed disease and were euthanased by day 16 PI. All four flying foxes seroconverted but failed to develop any clinical signs of disease. No gross lesions were observed at necropsy, but sub-clinical vascular lesions were seen, predominantly in the spleen. Virus was isolated from the two flying foxes killed at ten days PI (from heart and buffy coat of one, and from the spleen and kidney of its foetus, and from the kidney, heart and spleen of the second). No virus was isolated from the animals killed at 21 days PI, prompting the authors to conclude that the viraemic period in flying foxes may be short. The absence of virus recovery from the mouth, nose, rectum or urine further prompted the authors to suggest that excretion from these routes may be of little importance. Positive immunostaining of placental veins in the flying foxes killed at 10 days PI, and the recovery of virus from one of the foetuses demonstrated the possibility transplacental transmission. These findings support epidemiological evidence that will be presented in Chapter four.

2.2.5 Summary

Hendra virus is a novel paramyxovirus. It is zoonotic, has a wide host range, and is phylogenetically distinct. It has a high case fatality rate in horses and humans but is not highly infectious in these species. Experimental studies support flying foxes as the putative natural host of Hendra virus. The epidemiological evidence and the experimental studies support a broad hypothesis of the mechanism of spillover to horses as follows: (i) excretion from flying foxes, (ii) contamination of pasture, and (iii) ingestion by a susceptible horse. Intuitively, because spillover is a rare event, one or more of the factors contributing to attainment of *sufficient* cause for spillover must be obscure, or the combination of factors exceptional. The effective route of excretion from flying foxes, the periodicity of excretion, the means and length of survival outside the host, and other aspects of the ecology of the virus in flying fox populations is the subject of research presented in subsequent chapters.

2.3 NIPAH VIRUS

2.3.1 The emergence of Nipah virus

A major outbreak of disease in pigs and humans occurred in Peninsular Malaysia between September 1998 and April 1999 resulting in the death of 105 humans and the culling of over 1 million pigs (Chua *et al.*, 1999a; Nor *et al.*, 2000). Initially attributed to Japanese encephalitis virus, the primary disease aetiology was subsequently shown to be a previously undescribed virus of the family *Paramyxoviridae*. Preliminary characterization of an isolate at the CDC in Fort Collins and Atlanta, USA, showed the new virus, subsequently named Nipah virus, had ultrastructural, antigenic, serologic and molecular similarities to Hendra virus (CDC, 1999). Retrospective investigations suggest that Nipah virus has been responsible for disease in pigs in Peninsular Malaysia since late 1996, but the disease was not recognized as a new syndrome because the clinical signs were not markedly different from those of several endemic diseases, and because morbidity and mortality were not remarkable (Aziz *et al.*, 1999).

2.3.2 Natural infections in animals and humans

The epidemic primarily impacted pig and human populations, although horses, dogs and cats were also infected. The disease in pigs was highly contagious, and clinical disease was characterized by acute fever with respiratory and/or neurological involvement. Incubation was estimated to be 7-14 days. Crude case fatality rate was low (<5%), and notably, a

large proportion of infected pigs were asymptomatic. The clinical course appeared to vary with age. Sows primarily presented with neurological disease, and sows and boars sometimes died peracutely. In weaners and porkers, a respiratory syndrome predominated, frequently accompanied by a harsh non-productive (loud barking) cough. It is unclear whether respiratory and neurological symptoms observed in suckling piglets were directly attributable to infection. Epidemiological evidence suggests that the movement of pigs was the primary means of spread between farms and between regions (Nor *et al.*, 2000). The primary mode of transmission on pig farms was believed to be via the respiratory route; later laboratory evidence provided support for this contention (See Section 2.3.4).

The predominant clinical syndrome in humans was encephalitic rather than respiratory, with clinical signs including fever, headache, myalgia, drowsiness, and disorientation sometimes proceeding to coma within 48 hours (Chua *et al.*, 1999b; Goh *et al.*, 2000). The majority of human cases had a history of direct contact with live pigs. Most were adult male Chinese pig-farmers (Chua *et al.*, 1999a; Parashar *et al.*, 2000).

Evidence of infection has also been found in dogs, cats and horses (Chua *et al.*, 1999a; Nor *et al.*, 2000). The initially high prevalence of infection in dogs in the endemic area during and immediately following the removal of pigs suggests that dogs readily acquired infection from infected pigs. The much lower antibody prevalence and restriction of infection to within five kilometres of the endemic area suggests that Nipah virus did not spread horizontally within dog populations (Field *et al.*, 2001).

Malaysian bats became a surveillance priority in determining the origins of the virus because laboratory evidence suggested a close relationship between HeV and NiV, and because flying foxes had been shown to be a putative natural host of HeV. Neutralising antibodies to Nipah virus were found in 21 of 324 bats, from five of 14 species surveyed. These were the megachiropteran species *Pteropus vampyrus* (5/29), *P. hypomelanus* (11/35), *Cynopterus brachyotis* (2/56), and *Eonycteris spelaea* (2/38), and the microchiropteran species *Scotophilus kulhi* (1/33) (Johara *et al.*, 2001). Subsequently, Nipah virus was isolated from urine collected from a seropositive colony of *P. hypomelanus* on Tioman Island in Malaysia (Chua *et al.*, 2001).

2.3.3 Ultrastructural, molecular and phylogenetic studies of Nipah virus

Initial electron microscopic studies showed the ultrastructure of Nipah virus was consistent with that of viruses of the family *Paramyxoviridae*, and immunofluorescence tests of infected cells suggested a virus related to Hendra virus. Preliminary nucleotide sequencing also indicated that Nipah virus was related to Hendra virus (CDC, 1999) Virus particles were pleiomorphic, ranging from 120 to 500nm, and enveloped. Surface projections on the envelope measured 10nm. Typical 'herringbone' nucleocapsid structures were seen, approximately 1.67μm in length and 21nm wide. Nipah virus-infected cells reacted strongly with Hendra virus antiserum, but not other paramyxoviruses including measles virus, respiratory syncytial virus, and parainfluenza 1 and 3. No reactivity was seen with other viruses including herpes virus, enteroviruses and Japanese encephalitis virus (Chua *et al.*, 2000). Cross-neutralisation studies have shown at least a four-fold difference in neutralising antibodies between HeV and NiV (Chua *et al.*, 2000; Johara *et al.*, 2001).

Later, more extensive nucleotide sequence studies found that the nucleoprotein (N), phosphoprotein (P), and matrix (M) gene of Nipah virus shared a 70-78% nucleotide homology with Hendra virus, supporting the findings of others (see section 2.2.3) that HeV and NiV are phylogenetically closer to each other than to any other viruses in the subfamily *Paramyxovirinae* (Chua *et al.*, 2000; Harcourt *et al.*, 2000).

2.3.4 Experimental infections in animal species

The BSL4 classification of Nipah virus restricts experimental studies in Australia to the PC4 animal house facility at AAHL. AAHL has undertaken studies in pigs, cats and flying foxes. The study in pigs sought to describe clinical and pathological features of infection, and the mode of transmission of infection (Middleton *et al.*, 2002). Three of four six-week old pigs in one group were inoculated subcutaneously with 50000 $TCID_{50}$ of a non-plaque purified low-passage human isolate (s/c group). Three of four pigs in a second group were given the same dose of inoculum orally (p/o group). The fourth pig in each group acted as an in-contact control. Pigs were observed daily, and samples (blood, and tonsillar, conjunctival, nasal, rectal and urethral swabs) taken every second day for the 21-day duration of the trial. All pigs were clinically normal for the first six days PI. Over days 7-10, two of the s/c group became febrile and exhibited neurological signs including semi-

consciousness and lateral recumbency, or reluctance to rise and ataxia with diffuse muscle fasciculations. These pigs were euthanased because of animal welfare considerations. The third pig of the s/c group became depressed, had a mild temperature rise and a mucoid nasal discharge, shivered, and coughed persistently when disturbed. These signs regressed over several days. The in-contact pig in the s/c group was clinically depressed on day 9 but was otherwise clinically normal. Virus was isolated from the blood and tonsillar swab of one of the euthanased pigs, and from the nasal and tonsillar swabs of the in-contact pig on day 10. At necropsy, virus was isolated from the lungs, tonsil or spleen of both euthanased pigs, but not from the surviving two that were euthanased at day 21. Both of these pigs had seroconverted. In the p/o group, all pigs (except one of the inoculated pigs which died of an adverse reaction to the drug used for chemical restraint) were clinically normal throughout the study period. Virus was isolated from the tonsillar and/or nasal swabs of the surviving two inoculated pigs on days 4, 6, and 8, and from the in-contact pig on day 8. No virus was isolated at necropsy at day 21, but all (surviving) pigs had seroconverted.

In the second trial (Middleton *et al.*, 2002), two cats were given 50,000 TCID^{50} by the oronasal route. Both developed febrile illness on day 6 PI. Cat 1 was euthanased in respiratory distress on day 9. Virus was isolated from the urine of this cat on day 8. Cat 2 recovered, seroconverting by day 14. Virus was isolated from the tonsil and urine of this cat on day 2, from the tonsil on day 4, and from the tonsil, urine and blood on day 8.

In the third study (Daniels *et al.*, 2001b), 6 adult *P. poliocephalus*) were given 50,000 TCID^{50} by intramuscular inoculation. Samples (blood, bladder urine and ocular, nasal, tonsillar and rectal swabs) were taken every two days until days 21 –22 PI. All flying foxes remained clinically normal, and all seroconverted. Virus was isolated from the urine of one flying fox on days 12, 16 and 18.

2.3.5 Summary

Nipah virus is the second novel paramyxovirus linked to flying foxes. It is zoonotic, has a wide host range, and is phylogenetically close to Hendra virus. Unlike Hendra virus in horses, Nipah virus is highly infectious in pigs, with a high proportion of infected pigs subclinically infected. The case fatality rate in humans is high, as with Hendra virus. The available evidence suggests that Hendra and Nipah are ancient viruses, well adapted to their natural hosts, and in whose populations they have long circulated. The close

phylogenetic relationship between Hendra and Nipah viruses is consistent with a common progenitor virus. Does it follow that the natural histories of the two viruses are related? Does Nipah virus cycle in Australian flying foxes or does the presence of established cross-neutralising HeV infection preclude the establishment of Nipah virus in Australian populations? Experimental studies certainly demonstrate that Australian flying foxes are capable of maintaining Nipah infection in the absence of clinical disease. Investigation of the natural history of both viruses seems necessary to fully describe either.

2.4 AUSTRALIAN BAT LYSSAVIRUS

2.4.1 The emergence of ABLV

Australia has historically been considered free of rabies and the rabies-like viruses. Rhabdoviruses from the genus *Ephemerovirus* were known to occur (Bovine ephemoral fever, Adelaide River virus, Berrimah virus), but none from the genus *Lyssavirus* had been described. St. George (1989), postulating the origins of Adelaide River virus (which is antigenically related to rabies) had suggested the possibility of an undiscovered rabies-like virus in Australian bats in 1989. St. George went further, suggesting that the typically low prevalences of the rabies-related viruses in bats meant that an Australian bat lyssavirus might not become evident unless active surveillance of bats was undertaken, or unless man or a domestic animal were infected by a bat.

After wildlife surveillance for the origins of Hendra virus identified flying foxes as a natural host (see Chapter 3), the intensity of surveillance of bats in Australia increased substantially as researchers sought to explore the ecology of this virus. Thus, in May 1996, just a few months after the discovery of anti-Hendra antibodies in Queensland flying foxes, evidence of a lyssavirus infection was found in a flying fox in northern New South Wales (Fraser *et al.*, 1996) that was submitted for Hendra virus screening.

2.4.2 Natural infections in animals and humans

Natural infections of Australian bat lyssavirus have only been recorded in bats and in humans. No evidence of infection has been found in terrestrial carnivore species. There are several documented accounts of observed clinical disease in naturally infected flying foxes. Field *et al* (1999) present a case report describing a nine-day clinical disease course in an orphaned juvenile male black flying fox (*Pteropus alecto*) that was being hand-

reared by a volunteer wildlife carer. The bat had been in good health until its sixth week in care, when it suddenly began to exhibit signs indicative of neurological disease. On day 1 of illness, the bat developed sudden and progressive aggression towards its companion bat, and had repeated lordotic spasms during which it vocalized loudly. On day 2, the bat was calmer but still vocal, attempting to bite objects but eating little. On day 3, it was no longer aggressive and was only able to eat pulped food and milk. On day 4, it was seen by a veterinarian, who noted severe pharyngitis, and administered dexamethasone by injection. The bat was much more alert that evening and ate solid food well. The dexamethasone injection was repeated on day 5; the bat remained alert and ate solid food overnight. On day 6, it was dysphagic and was again offered pulped foods and liquids. On days 7 and 8, it was unable to roost normally, lay supine, was progressively dysphagic, had diarrhoea, and was losing weight. On day 9, it rapidly deteriorated and died. On necropsy, sections of brain showed nonspecific, nonsuppurative meningoencephalitis with perivascular cuffs of mononuclear cells and widespread focal gliosis. Numerous neurons contained eosinophilic inclusion bodies, highly suggestive of lyssavirus infection. ABLV infection was diagnosed by FAT on impression smears of fresh brain. Immunoperoxidase staining of formalin-fixed, paraffin-embedded sections of brain detected lyssavirus antigen in neurons of the frontal cortex, hippocampus, brain stem, and cerebellum, including Purkinje cells. The authors argued that, as natural in-utero infection with lyssaviruses is not known to occur, the bat was infected in the 2-3 weeks between it's birth and when it came into care. All four flying foxes, which had been in contact with the case animal while in care subsequently, tested negative for ABLV antibody and antigen. This reasoning indicates an incubation period of 6 to 9 weeks in this case.

A second clinical case report (HE Field *et al*, in preparation) details an observed 24-hour clinical course in a captive adult female *P. poliocephalus*. The animal was one of a captive colony of 25 flying foxes in central Queensland. Observations noted by the carers are presented in Box 2.1. Approximately one month prior to this incident, a debilitated wild flying fox found on the roof of the captive colony enclosure tested positive by FAT for ABLV antigen in a fresh brain smear. It is believed that this bat was the probable source of infection for the colony bat. This incident highlights several issues in relation to the safe maintenance of captive flying fox colonies, particularly those on public display. In particular, appropriate measures to preclude direct contact between wild and captive flying foxes, and consideration of vaccination of captive flying foxes. The former can be achieved by means of appropriate exclusion netting over the roof and walls of the

enclosure. The latter is more problematic, having both policy and practical impediments. At present, rabies vaccination for veterinary use in Australia is at the discretion of the chief veterinary officer in each state. Further, knowledge of the efficacious use of vaccination in flying foxes is limited. While Barrett (2004) showed that flying foxes vaccinated with a commercial rabies vaccine developed antibody titres equal to or greater than 0.5 IU/ml within 28 days, post-vaccination challenge experiments with ABLV were not undertaken. However, the author reasonably argues that the presence of cross-reacting neutralising ABLV titres equal to or greater than 1:154 in vaccinated flying foxes with rabies RFFIT titres equal to or greater than 2 IU/ml indicates likely protection against ABLV.

Box 2.1: Carer observations of the clinical course of Australian bat lyssavirus infection in a grey-headed flying fox.

22/11/00

- 10.00am: observed to be hanging alone, 'hunching up', and vocalizing as if in pain.
- 10.30am-1.00pm: continually licking vulva and perineal region.
- 1.00pm: veterinary examination inconclusive; a tentative diagnosis of cystitis made, antibiotics and non-steroidal anti-inflamatories prescribed.
- 2.00-400pm: not her normal withdrawn self; taking pieces of fruit aggressively; vocalization (in the absence of any observed spasms), agitation and aggression getting progressively worse.
- 4.00pm: moved to another carer; very agitated and very vocal.
- 6.00pm: a little quieter, licking at her vulva, frequent muscle spasms; attacking her food bowl.
- 7.30pm: agitated, seems very stiff but still moving about the cage.
- 7.40pm: having spasms; no longer hanging from the top of the cage.
- 8.00pm: moving slowly around the cage; has salivated or urinated on herself; eyes glassy and moist; seems in great pain; vocalizing a little and fanning her wings as if hot; still biting her food bowl as she passes it.
- 8.30pm: seems to be having a seizure; strange vocalization, salivating profusely, tears streaming.
- 9.00pm: another seizure; not as vocal but shaking violently.
- 10.00-11.00pm: two further seizures; very vocal.

23/11/00

- 6.00am: almost comatose; euthanased and forwarded for diagnostic testing.

In late October 1996, six months after its first description in a flying fox, the zoonotic potential of ABLV was demonstrated when a 39-year-old female wildlife carer in central Queensland developed a fatal neurological illness (Allworth *et al.*, 1996). The woman had been caring for several black flying foxes and an insectivorous yellow-bellied sheath-tailed bat (*Saccolaimus flaviventris*) in the period prior to her illness. Presenting signs were pain and numbness in the left arm, progressing over the next 2-3 days to fevers, headaches, dizziness and vomiting. She was admitted to hospital where, despite treatment, her condition deteriorated. Over days 8-10, the woman developed complete extraocular muscle palsies, progressive weakness in all limbs and eventually a depressed clinical state. By day 11, she was areflexic, unresponsive,

hyperthermic and ventilator-dependent. Serum tested positive for anti-lyssavirus antibodies, and a PCR test on cerebrospinal fluid was positive to Australian bat lyssavirus specifically. There was a history of numerous scratches (but not of bites) from the flying foxes in the two to four weeks preceding her illness (Allworth *et al.*, 1996), and a history of a bite from the yellow-bellied sheath-tail bat six weeks prior to the onset of clinical signs (Speare *et al.*, 1997). Subsequent antigenic and genetic evidence supported a bat of this species being the source of infection (Gould *et al.*, 2002; Gould *et al.*, 1999).

A second human case of lyssavirus disease occurred in 37-year-old woman in central Queensland in late November 1998 (Hanna *et al.*, 2000). The woman died after an apparent prolonged incubation period: the only history of contact with bats was 27 months earlier when she was bitten on the finger while removing a flying fox which had landed on the back of a child. On presentation, there was a five-day history of fever, vomiting, anorexia, parathesia of the dorsum of the left hand, pain about the shoulder, a sore throat and difficulty swallowing. Deterioration, instanced by increased agitation, dysphagia, and dysphonia, and frequent and severe muscular spasms was evident 12 hours later. A diagnosis of ABLV was considered at this point. From day 2, the patient was apparently unable to understand verbal commands or to communicate. She remained ventilator-dependent from this point, exhibiting purposeless movements (facial grimacing, rolling eye movements) and muscular spasms (arching of the back) whenever the dose of muscle relaxant was reduced. On day 4, an initial PCR result on saliva indicated an ABLV-specific product. This was subsequently confirmed, and additionally, molecular sequencing identified it as the flying fox variant of ABLV. The remarkable feature of this case is the prolonged incubation period. The usual incubation period for rabies is generally accepted as 20-90 days, with 95% of cases occurring within a year of exposure (Hanna *et al.*, 2000). Notable also in this case is that the exposure event occurred within several weeks of the first fatal human case.

ABLV has not been reported in any other species than bats and humans. It seems improbable that terrestrial predators such as feral cats and foxes, known to scavenge under flying fox camps, have not been exposed to bats debilitated or moribund with terminal ABLV infections. Have there been sporadic undiagnosed cases in these species?

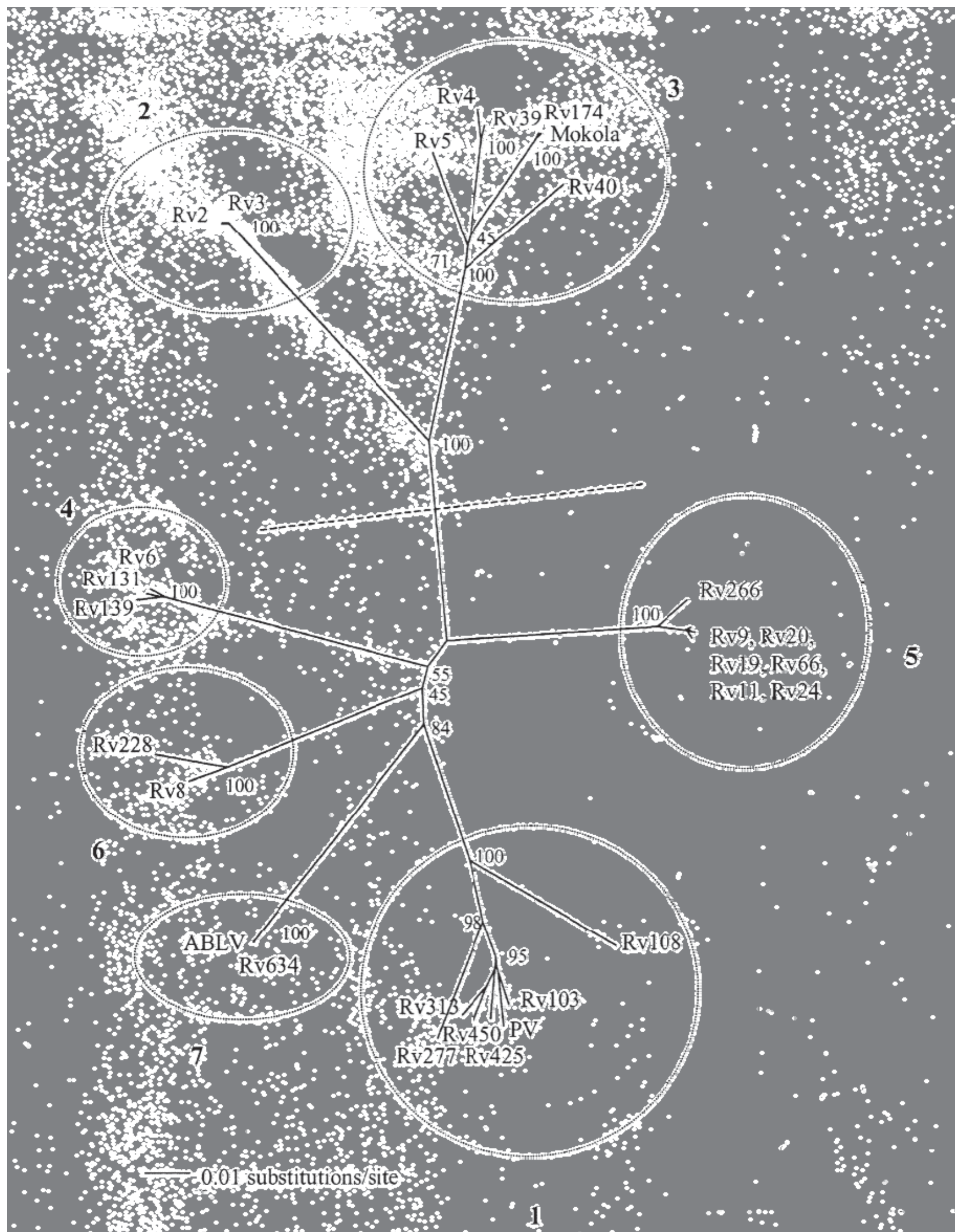
2.4.3 Molecular and phylogenetic studies of Australian bat lyssavirus

Lyssaviruses belong to the family *Rhabdoviridae*. Prior to the description of ABLV there were six recognized species (genotypes) in the genus: classical rabies virus (genotype 1), Lagos bat virus (genotype 2), Mokola virus (genotype 3), Duvenhage virus (genotype 4), European bat lyssavirus 1 (genotype 5) and European bat lyssavirus 2 (genotype 6). The ICTV has recognized Australian bat lyssavirus as a new species (genotype 7) (ICTV, 2000). While the seven genotypes are genetically distinct, some serologic cross-reactivity does occur, evidenced by the existence of only four serotypes. Further, the seven genotypes have recently been aggregated into two phylogroups, based on whether they are pathogenic in mice by both intracerebral and intramuscular routes (phylogroup I) or only by the intracerebral route (phylogroup II) (Badrane *et al.*, 2001). Figure 2.3 presents a phylogenetic tree of the genus.

ABLV is closely related to classical rabies virus (CRV). Gene sequence analysis of the ABLV nucleocapsid (N) protein gene (used as the basis for genotypic classification) showed marked nucleotide and amino acid homology (73-74% and 92% respectively) with classical rabies viruses. Gene sequence analysis of the ABLV phosphoprotein (P), matrix (M) protein and glycoprotein (G) also revealed a closer sequence homology to classical rabies viruses than to the other lyssaviruses (Gould *et al.*, 1998). Additionally, ABLV and CRV share the same serotype (1), with cross-neutralisation evident against 19 of a panel of 21 monoclonal antibodies (Gould *et al.*, 1998). Rabies vaccine and antirabies immunoglobulin have been shown to protect laboratory animals from ABLV infection (Hooper *et al.*, 1997). These features have triggered robust discussion on the validity of ascribing ABLV to a new genotype.

Two variants of ABLV have been described, both belonging to genotype 7. Isolates from flying foxes and from yellow-bellied sheathtailed bats are able to be readily distinguished into two distinct clades by sequence analysis (Gould *et al.*, 2002; Gould *et al.*, 1999).

Figure 2.3: Phylogenetic relationships between isolates of the genus *Lyssavirus* based on the glycoprotein coding sequence[1].



The bold numbers outside the circle refer to genotype; the 'Rv' numbers inside the circle refer to isolate. The dashed line shows the separation of phylogroups proposed by Badrane *et al* (2001). The relationships are presented as an unrooted maximum likelihood phylogram. Bootstrap values are shown within the figure, with values of over 70% considered significant.

[1] Adapted from Johnson *et al* (2002)

2.4.4 Experimental infections in animal species

Limited experimental studies have been undertaken with ABLV. McColl (McColl *et al.*, 1999; McColl *et al.*, 2002) describes pathogenesis studies in flying foxes comprising two trials undertaken at AAHL. In the first trial, seven wild-caught *P. poliocephalus* were inoculated intramuscularly in the forelimb with $10^{3.7}$ $TCID_{50}$ of ABLV, seven were inoculated with $10^{3.3}$ $TCID_{50}$ of a bat-derived rabies virus, and one remained as an uninoculated control. Intracerebrally inoculated mice were used as inoculum controls. Blood samples were taken prior to inoculation, and blood and tissue samples were taken at necropsy two months post-inoculation. All 15 animals were negative for anti-lyssavirus antibodies at the commencement of the trial. At day 27 PI, one of the ABLV-inoculated bats became obtundent and developed severe muscle weakness. It was diagnosed lyssavirus-positive by FAT on brain impression smears, by histopathology, and by immunoperoxidase staining. None of the other 13 inoculated bats exhibited clinical signs, none showed evidence of infection on post-mortem examination, and none seroconverted. All inoculum control mice were symptomatic and euthanased at day 9-10 PI. Lyssavirus presence was demonstrated by FAT. In the second trial, 15 grey-headed flying foxes were sourced from a captive colony. This was an attempt to avoid biosafety-related restrictions to the study design of trial one when seven of the 15 wild-caught animals were found to have a positive neutralising antibody titre to Hendra virus at the start of the trial, and a further three had seroconverted by the end of the trial. Ten animals were inoculated intramuscularly in the forelimb with 10^5 $TCID_{50}$ of ABLV, four were inoculated with an equal dose of bat-variant rabies, and one remained an uninoculated control. Blood, saliva, rectal and (less regularly) urine samples were collected regularly over a three-month period. Three of the 10 ABLV-inoculated bats developed clinical signs including muscle weakness, ataxia, paralysis or paresis at days 15, 23 and 24 PI. Two of the four rabies-inoculated bats exhibited similar signs on days 16 and 17 PI. All five cases had histopathological lesions and were positive for viral antigen in brain impression smears by FAT. Four of five were positive by immunoperoxidase staining, and virus was isolated from three. The author suggests that the trials demonstrate a dose-dependent response. While this is a biologically plausible interpretation, it should be noted that there is no statistically significant difference (p=0.05) between disease prevalence in the two trials.

Barrett (2004) also undertook experimental infections in flying foxes. Nine pre-breeding or young adult seronegative *P. poliocephalus* were inoculated with 0.12ml of a 20% weight per volume suspension of salivary gland from a naturally infected *P. alecto*. The inoculum

contained $10^{5.2}$ to $10^{5.5}$ $MICED_{50}$, and was injected equally at four sites: the left 'footpad', left pectoral muscle, left temporal muscle, and left muzzle. A tenth individual received only 0.06 ml of the inoculum, divided between the left footpad and the left pectoral muscle. Seven of the 10 flying foxes (including the animal that received the lower dose) developed clinical disease of one to four days duration between 10 and 19 days PI, and either died or required euthanasia. Infection with ABLV was confirmed in each symptomatic flying fox by FAT on fresh brain impression smear, detection of antigen in formalin-fixed tissues, virus isolation in mice, and Taqman® PCR. The three asymptomatic flying foxes remained clinically healthy until killed on day 80 or day 82 PI, at which time they were negative for ABLV by FAT, immunohistochemistry and mouse inoculation. That these three flying foxes, which received the higher inoculum dose, were uninfected, when the individual receiving the lower dose succumbed to infection argues against a simple dose-related response as suggested above by McColl. Alternate plausible explanations for the apparent absence of infection in these individuals include incubation beyond 82 days, and immunity independent of detectable neutralising antibodies.

McColl (1999; 2002) also undertook experimental susceptibility studies in cats and dogs. In the cat trial, three adult cats were inoculated with 10^{5} $TCID_{50}$ of ABLV into the forelimb musculature, one was inoculated with a similar dose of bat-variant rabies, and one remained as an uninoculated control. Clinical samples including blood and oral swabs were taken regularly over a three-month period. Behavioural observations were recorded daily. No cats exhibited clinical disease, however the three ABLV-infected cats were found to have seroconverted (at days 29, 42 and 95 PI). Anti-lyssavirus antibodies were found in the CSF of one cat. All inoculated cats were observed to exhibit mild, transient behavioural abnormalities at some time between 11 and 42 days PI. This trial suggests that ABLV causes little or no clinical disease in infected cats, however as the author notes, this interpretation is clouded by the absence of clinical disease in the bat-variant rabies inoculated cat. The latter had previously been found to cause fatal disease in four of four inoculated kittens within 25-60 days (Trimarchi *et al.*, 1986). Was age a factor? Was the trial of insufficient length? Was the study design inadequate? Inoculum control mice were euthanased 5-8 days PI, and lyssavirus presence confirmed by FAT.

In the dog trial, three pups were inoculated with a dose of $10^{3.7}$ $TCID_{50}$ of ABLV, two were inoculated with 10^{5} $TCID_{50}$ of ABLV, two were inoculated with 10^{5} $TCID_{50}$ of a bat-variant rabies, and two remained as uninoculated controls. Both pups inoculated with bat-variant

rabies developed clinical disease and were euthanased at days 9 and 12. Of the five ABLV-inoculated pups, three developed mild transient clinical signs and recovered. All five seroconverted; two had antibodies in the CSF. Histopathology on the brain and spinal cord was negative for all ABLV-inoculated pups but positive for both bat-variant rabies inoculated pups. The outcomes of this trial suggest that infection with ABLV in pups causes little or no clinical disease, despite evidence of virus reaching the CNS. The author notes that Tignor *et al* (1973) obtained similar negative results using Mokola and Lagos bat virus in dogs. With the exception of vampire bat-vectored rabies, reports of the natural transmission of bat lyssaviruses to terrestrial animals have been uncommon. Is this because infection or disease as a result of exposure to bat lyssaviruses is improbable in the terrestrial spillover hosts, or, as McColl *et al* (2000) suggest, because animal isolates are rarely typed to identify their origin?

2.4.5 Summary

Australian bat lyssavirus is a previously undescribed lyssavirus. Genetically related to (but distinct from) classical rabies virus, it shares the same serogroup as CRV. Rabies vaccination provides protection. Natural infections have been recorded only in bats and in humans. Distinct variants have been demonstrated in the two sub-orders of bats in Australia, suggesting that the virus is not a recent introduction to Australian bat populations. The occurrence and frequency of infection in bat species and populations across their Australian range is unknown. The dynamics of infection in flying fox populations is unknown. Does it parallel bat-variant rabies elsewhere or are there important differences? Is it possible to rule out established infection in terrestrial carnivores?

2.5 THE BIOLOGY OF FLYING FOXES (*Pteropus* spp.)

2.5.1 Introduction

There are about 925 species of bats (order *Chiroptera*) worldwide, divided into the suborders *Megachiroptera* (42 genera and 166 species) and *Microchiroptera* (135 genera and 759 species). Generally, microchiropterans are small insectivorous bats that navigate by echolocation. Australia has six families of microchiroptera, 20 genera and about 63 species. Some genera are restricted to Australia. Megachiroptera are generally larger

fruit-eating bats that navigate by sight. There is just one family (*Pteropodidae*), which is represented in Australia by five genera and 13 species (Hall & Richards, 2000).

2.5.2 Global distribution and general biology of *Pteropus* species

The bats commonly known as flying foxes belong primarily to the genus *Pteropus*. There are 65 species of *Pteropus* worldwide. The distribution of flying foxes extends from the West Indian Ocean islands of Mauritius, Madagascar and Comoro, along the sub-Himalayan region of India, Pakistan and Nepal, through southeast Asia, The Philippines, Indonesia, New Guinea, the islands of the western Pacific ocean (as far east as the Cook Islands) and Australia (excluding Tasmania). Based on maximal species diversity, flying foxes are believed to have originated from Sulawesi and eastern New Guinea, where up to six species occur. Many species are restricted to islands, but a number are widespread. Flying foxes have the largest body size of all bats, ranging from 300gms to over 1kg, and in wingspan from 600mm to 1.7m. Females usually have only one young a year after a 6-month pregnancy. The young grow rapidly but are dependent on their mother for up to 3 months. Flying foxes are nocturnal mammals. They form very visible and noisy daytime ‘camps’ where they collectively hang in trees. Camps can be in mangroves, swamps, rainforest or tall mixed forest, and are commonly beside water (Corbett & Hill, 1986; Hall & Richards, 2000; Mickleburg *et al.*, 1992; Nowak, 1994).

2.5.3 The biology of Australian flying foxes

2.5.3.1 Distribution and occurrence

There are seven species of flying foxes recorded for Australia. One is probably extinct (*Pteropus brunneus*), and two are restricted to the islands of Torres Strait (*P. banakrisi* and *P. macrotus*). Four species are found on the Australian mainland: the grey-headed flying fox (*P. poliocephalus*), the black flying fox (*P. alecto*), the spectacled flying fox (*P. conspicillatus*) and the little red flying fox (*P. scapulatus*) (Figure 2.4). The grey-headed flying fox is the only species restricted to Australia, occurring from Melbourne along coastal eastern Australia to Bundaberg in southern Queensland. The black flying fox, found in Australia from the mid-New South Wales coast north along coastal Queensland, Northern Territory and Western Australia (down to Carnarvon), also occurs in New Guinea and parts of Indonesia (Irian Jaya, Lombok, Sulawesi). The spectacled flying fox, in Australia restricted to the (northern) wet tropics of Queensland, is also found in New Guinea and on the Indonesian island of Halmahera. The little red flying fox, recorded over

a large part of the eastern, northern and western parts of the Australian continent also occurs in New Guinea (Hall, 1987; Mickleburg *et al.*, 1992).

Figure 2.4: The distribution of species of flying foxes on mainland Australia[1]

Key: Horizontal hatching = *P. alecto*
Vertical hatching = *P. poliocephalus*
Solid black = *P. conspicillatus*
Broken line = southern inland limit of *P. scapulatus*.

[1] Adapted from Hall and Richards (2000).

2.5.3.2 Movement patterns and flying behaviour

Flying foxes commonly fly at 25-30 kilometres per hour, sustainable for several hours. Flying height varies with topography but is generally 30-40 metres when foraging, and 60-80 metres over long distances. However little red flying foxes in particular will fly close to the ground over flat open country and over water (Hall & Richards, 2000).

The movement patterns of flying foxes are primarily governed by the availability of food. Little red flying foxes, whose food preference is the nectar of eucalypt flowers (rather than fruit), undertake seasonal movements that are correlated with the flowering of favoured food trees. These migrations may be over hundreds of kilometres, with numbers in the tens or hundreds of thousands of animals. The regularity of the flowerings, and hence the migrations, is dependent on climatic and anthropogenic variables. Where rainfall is less dependable, or where land-use changes have removed historically utilized food sources, the seasonal movements become more nomadic in nature. The grey-headed, black and spectacled flying foxes (either dietary generalists or frugivorous) tend to have more localized movement patterns (Hall & Richards, 2000). Webb and Tidemann (1996) recorded an average distance of 250 kilometres traveled between banding and recovery of 10 marked grey-headed flying foxes over 15 months. Notwithstanding, one of the animals traveled nearly 1000 kilometres in five months. Radiotelemetry studies led Eby (1991; 1995) to suggest that grey-headed flying foxes may make regular movements of hundreds of kilometres. Such a pattern of movement, repeated across the species range, would mean a population in a constant state of flux. Genetic studies in grey-headed, black and little red flying foxes support such a theory (Sinclair *et al.*, 1996; Webb & Tidemann, 1996). Low indices of among-population variation were found for all three species, consistent with an homogenizing action of movements across species ranges. Observations on flying fox movements in Torres Strait (between Australia and New Guinea) showed a yearly movement cycle which involved flying foxes moving into the islands from New Guinea, down to Cape York and back into New Guinea. Two species, the black and large-eared were the principal flying foxes involved in these movements (Hall & Richards, 2000). The large-eared flying fox is also found on islands along the southern coast of New Guinea and several nearby Indonesian islands. The possibility of movements of flying foxes between New Guinea and Indonesian islands and onto Southeast Asia has never been studied. There is however, anecdotal evidence that flying foxes can cross large distances over water, albeit inadvertently. There is record of a little red flying fox in New Zealand, 1600 kilometres from its southernmost Australian occurrence, and a sighting of Indian

flying foxes (*P. giganteus*) 350 kilometres from land (Sinclair *et al.*, 1996). Both reports are believed to be of animals that have been blown off shore by strong winds.

It is noteworthy that the overlapping distributions of only three species of flying foxes are needed to form a continuous link between the east coast of Australia and Pakistan. Black and Spectacled flying foxes are known to overlap with the Island flying fox (*P. hypomelanus*) and the Malayan flying fox (*P. vampyrus*) in New Guinea and Indonesia, and these species, at the northern extent of their range, overlap with the Indian flying fox, whose distribution extends eastward (from Thailand and Burma) across to India and Pakistan (Corbet & Hill, 1992; Mickleburg *et al.*, 1992).

2.5.3.3 Reproductive behaviour and lifecycle

Flying foxes are seasonal breeders with mature females producing one young per year. Sexual maturity is reached in the second or third breeding season (at 18-30 months of age). The peak mating period for grey-headed, black and spectacled flying foxes is April-March, although latitude is reported to influence the timing and duration of the breeding season, with a more extended breeding season apparent in northern Australia (Hall, 1995). Gestation is about six months, with most grey-headed, black and spectacled flying foxes giving birth from late September to November. Males and females tend to segregate at this time. Birth normally occurs in the camp during daylight hours. The placenta is eaten. From the age of about three weeks, the young are left in communal creches in the camp at night while the mother forages. By six weeks they tend to remain at their mothers roosting site while she forages. Young bats can fly at the age of three months, but are still maternally dependent. In January and February, the young flying foxes form small groups in the camp. Males tend to roost away from females during rearing (September to January), and by February-March, (leading up to the mating season) have begun to establish territories that they aggressively defend. After the end of April, males become less aggressive, cease to defend their territories and return to mixed groups (Hall & Richards, 2000).

The peak mating period for the little red flying fox is November-December, six months out of phase with the other species. Gestation is again six months, with most little red flying foxes giving birth in May and June (Hall & Richards, 2000).

2.5.3.4 Camp location and roosting behaviour

Some camps are permanent, while others are seasonal, the primary criterion being the availability of food. Even within permanent camps, the numbers and class of animals present over the year may reflect food availability or life cycle stage. From mid-April to September, major population shifts occur; large summer camps fragment and move, and transient camps take advantage of local food abundance. A trend of increasing urbanization of flying foxes, particularly in eastern Australia, has been observed throughout the 1980s and 1990s. This has been attributed to a reduced availability or suitability of historic sites because of land-use changes, and a concurrent increase of reliable food sources in well-watered suburban gardens (Hall & Richards, 2000).

Flying foxes hang upside down on branches in their camps. The roosting density varies with species and with the stage of the life cycle. While grey-headed, black and spectacled flying foxes commonly distance themselves about one body space from their neighbour. Little red flying foxes typically crowd together, frequently forming dense clusters of 20 or 30 animals. Where more than one species of flying fox occurs, camps are generally shared. In northern Australia, black and spectacled flying foxes share camps with each other, and in New Guinea and Indonesia, with non-Australian species (Hall, 1987; Waithman, 1979).

2.5.4 Summary

Bats are ubiquitous animals. Bat species represent almost 25% of all known mammalian species (McColl *et al.*, 2000). Flying foxes are highly mobile mammalian species. Camps and populations are dynamic. There is evidence of flying fox populations under stress from anthropogenic changes and of niche expansion into urban areas. Are these factors associated with the emergence of previously unknown viruses?

2.6 METHODOLOGIES FOR INVESTIGATING WILDLIFE RESERVOIRS OF DISEASE.

2.6.1 Introduction

The effective implementation of studies of wildlife populations frequently presents challenges that are not encountered in domestic species. Many of these challenges stem from the uncontrolled nature of wildlife populations where basic population parameters are generally unknown. Additional constraints may be posed by a limited knowledge of the host species biology, invalid or inadequate diagnostic tests, statutory protection afforded by the species conservation status, and public opposition by individuals or groups. Thus methodologies commonly applied to domestic animal populations may or may not be appropriate for wildlife studies.

2.6.2 Observational study methodologies

In the broadest context, epidemiological studies may be experimental, observational or theoretical: the choice of methodology largely depends on the objective of the study. Studies of the ecology of a disease commonly have as objectives the elucidation of the distribution of disease in a population, the mode of transmission of infection, the mechanism whereby infection is maintained in the population, and factors that are causally associated with disease. Because observational studies record the pattern of infection or disease in a target population in its natural environment, they are a key methodology in the study of wildlife reservoirs of disease. Such studies are frequently partly descriptive, partly analytical, and may be hypothesis-forming and/or hypothesis-testing.

Three types of observation study are traditionally described: cross-sectional studies, case-control studies, and cohort studies. Cross-sectional studies typically select a random sample of a target population, and (in the context of a disease investigation) record the presence or absence of disease and of putative causal factors for each individual. From these data, the extent of any association between disease prevalence and putative causal factors can be determined. One of the major advantages of a cross-sectional study design is the ability to simultaneously study many factors and many diseases, and to derive direct estimates of their frequency in the target population. They can be relatively inexpensive and quick studies, and are commonly used in studies of disease in wildlife populations. Cross-sectional studies have two major limitations; the inability to provide a direct

measure of disease incidence, and the large sample size required to meaningfully investigate rare diseases or diseases of short duration (Martin *et al.*, 1987; Thrusfield, 1986). With respect to the former, an estimate of disease incidence is possible when the duration and prevalence of the disease is known (Thrusfield, 1986). Also, McGowan *et al* (1992) describes a novel method of estimating annual incidence using age-specific prevalence rates. Depending on the species and the sampling interval, a series of cross-sectional studies is sometimes able to provide useful temporal data. Morris and Pfeiffer (1995) caution that cross-sectional studies can suffer poor internal validity as a result of an inadequate or unrepresentative sample. They cite bias of unknown severity that results from unequal catchability of different classes of animals. Notwithstanding, the potential for sampling bias in wildlife studies is not restricted to cross-sectional designs (Martin *et al.*, 1987; Thrusfield, 1986).

In a typical case-control study, separate samples of animals with the disease of interest (cases) and without the disease of interest (controls) are selected. The relative frequency of each factor in each group is compared, allowing factors associated with disease to be identified and the strength of association measured. Morris and Pfeiffer (1995) note that this methodology has not been widely used with wildlife populations, though it could be used to identify likely risk factors and to re-analyze cross-sectional study data. The major advantages of the case-control methodology are the ability to simultaneously screen multiple risk factors for a disease of interest, and its suitability in investigating rare diseases (Martin *et al.*, 1987; Thrusfield, 1986).

Cohort studies typically follow samples of animals with and without a factor of interest over time, and compare the rates of disease in each. The major advantages of cohort and other longitudinal studies are their ability to provide direct estimates of disease incidence in the target population, and to robustly demonstrate causal associations (Martin *et al.*, 1987; Thrusfield, 1986). Morris and Pfeiffer (1995) contend that longitudinal studies are the single most reliable technique for investigating wildlife disease. They favour a capture-mark-recapture methodology (Pollock *et al.*, 1990; Seber, 1982) that offers the opportunity of multiple observations of individuals over time, and the ability to estimate prevalence, incidence, and population size. The data also facilitates temporal analyses (using techniques such as survival analysis and Cox's proportional hazard regression analysis) and spatial analyses (using geographic information systems). In CMR studies, a sample of the study population is captured, uniquely identified (marked) and released. At

subsequent sampling periods, marked animals (and unmarked animals) are recaptured. One of two methodologies is then employed to calculate estimates of population parameters - either an enumeration method or probabilistic modelling. The latter, which calculates estimates using statistical theory, is generally considered to offer more accurate estimates (Jolly & Dickson, 1983; Nichols & Pollock, 1983). Basically, the method derives estimates from the ratio of the number of marked animals to the total number of animals captured in a sampling period *i+1.* Providing model assumptions are met, this sample ratio reflects that in the population. The model uses capture probabilities plus survival probabilities to estimate population size at each sampling, and to estimate survival rates and recruitment between samplings. Pfeiffer (1991) used a CMR design as the basis for an epidemiological study of bovine tuberculosis in brush-tailed possums (*Trichosurus vulpecula*) in New Zealand. An open population model was used to estimate population size, survival rates and birth rates. Animals categorized as tuberculosis-suspect were fitted with radio transmitters to enable the location of den sites, the calculation of home ranges, and the recovery of carcasses. A GIS was used to map the study area, record spatial data, and identify spatial patterns. However, longitudinal studies have a major limitation; they are costly in time and resources (Martin *et al.*, 1987; Thrusfield, 1986). Also, valid estimates from CMR studies are dependent on a substantial proportion of the population being marked, and on a substantial rate and frequency of recapture. The technique has been shown to be inefficient in flying fox population studies because the size of populations precludes the marking of an adequate proportion, and the mobility of populations precludes an adequate rate of recaptures. Webb and Tidemann (1996) report a recapture rate over 15 months of only 1.1% (10) of 943 marked grey-headed flying foxes.

Mills *et al* (1998) propose a series of consecutive but overlapping steps that include cross-sectional and longitudinal approaches in investigating infections in wildlife populations. These steps are

- i) Definition of the geographic distribution of the host.
- ii) Definition of the distribution of the agent within the host range.
- iii) Identification of any ecological variables influencing agent and host distribution.
- iv) Identification of significant host-level variables.
- v) Longitudinal studies to enable a temporal perspective.
- vi) The development of predictive models.

Defining host and agent distributions (steps i and ii) can be efficiently achieved with cross-sectional surveys (Mills *et al.*, 1998). Identification of putative risk factors (steps iii and iv) can equally be achieved using a case-control analytical approach on cross-sectional data or by a longitudinal study (Mills *et al.*, 1992; Mills *et al.*, 1997). A temporal pattern of infection or disease can only be obtained from a longitudinal study, in the form of either a series of cross-sectional studies or a CMR study (Mills *et al.*, 1992). The research presented in this thesis broadly follows the approach of Mills *et al* (1998).

Observational study designs frequently incorporate additional methodologies. Radiotelemetry, global positioning systems, and geographic information systems are commonly used in wildlife studies. These techniques enable the collection and analysis of spatial data. Experimental studies and modelling studies may complement observational studies. Experimental studies are often valuable in demonstrating probable modes of transmission and mechanism of maintenance of infection, albeit in an experimental setting. This information can then focus observational studies in the natural environment. Modelling studies can be similarly valuable either at a conceptual level, for simulation studies, or to facilitate the development of management strategies. Modelling is discussed further in Section 2.7.

2.6.3 Laboratory methodologies

2.6.3.1 Hendra virus diagnostics

Daniels *et al* (2001a) describe six diagnostic methodologies for the detection of Hendra virus infection: virus isolation, electron microscopy, immunohistochemistry, PCR and sequencing, serum neutralisation tests and ELISA. The first four methodologies detect virus, virus antigen or virus nucleotide sequence (that is, evidence of current infection); the latter two detect antibody (that is evidence of past infection). As discussed in Section 2.2.4, Hendra virus is classified internationally as a BSL4 agent. This classification has diagnostic ramifications in that tests necessarily involving live virus (virus isolation beyond primary diagnosis and serum neutralisation tests) in that such work should only be carried out under PC4 conditions after appropriate training.

Virus isolation

Daniels *et al* (2001a) comment that Hendra virus grows well in Vero cells from a range of tissue specimens including brain, lung, kidney and spleen. CPE usually develops within 3 days, initially manifest by the formation of syncytia containing 20 or more nuclei, and

subsequently by punctate holes in the cell monolayer. Virus isolates can be specifically identified by immunostaining, neutralisation with specific antiserum, PCR and electron microscopy. Virus isolation is an important diagnostic tool, and where appropriate protocols and training are in place, the authors suggest that primary virus isolation (for diagnostic purposes) can be performed in PC3 laboratories. Importantly, they caution that any cultures that develop characteristic CPE be forwarded to a PC4 laboratory for further work.

Immunohistochemistry

In contrast, because immunohistochemistry is performed on formalin-fixed tissues, the same biosafety constraints don't apply. Daniels *et al* (2001a) comment that IHC is a useful and safe technique that can detect virus antigen in a range of tissues. Indeed they recommend submission of a range of tissues (including spleen), noting that virus may clear from lung tissue early in an infection. They also note that because IHC uses formalin-fixed tissues, the technique is useful for retrospective investigations on archived materials. The availability of a range of polyclonal and monoclonal antisera means that test sensitivity and specificity can be tailored to testing objectives.

Electron microscopy

Negative contrast electron microscopy and immunoelectron microscopy were an integral part of the initial Hendra virus diagnostic effort (Murray *et al.*, 1995b). Daniels *et al* (2001a) recognize the value of both in providing rapid and valuable information on virus structure and antigenic reactivity during primary virus isolation, and see these and other EM techniques as complementing other diagnostic methodologies.

PCR and sequencing

PCR is a powerful molecular technique able to amplify segments of virus genes. The diagnostic PCR in routine use at AAHL employs a set of nested primers to amplify segments of the Hendra virus M gene that codes for the relatively conserved matrix protein (Daniels *et al.*, 2001a). The ability to select primer sets for various genes means that test sensitivity and specificity can be tailored to testing objectives. The technique can be used as a primary diagnostic tool to detect virus sequences in fresh or formalin-fixed tissue fixed or cerebrospinal fluid, or as an adjunct to virus isolation to rapidly characterize virus isolates. The inherent high sensitivity of PCR associated with the amplification process means that the risk of laboratory contamination and subsequent false-positive test results

is ever-present. This issue can largely be addressed by appropriate laboratory design, personnel training, and internal and external quality control programs.

Serum neutralisation tests

The serum neutralisation test is regarded as the 'gold standard' serologic test for Hendra virus. Sera are incubated with live virus in microtitre plates to which Vero cells are added. Initial serum dilutions of 1:2 or 1:5 can be used. Cultures are read at three days, and those sera blocking CPE are regarded as positive (Daniels *et al.*, 2001a). The use of live virus and the attendant biosafety issues means that SNT should only be performed in a PC4 facility.

ELISA tests

ELISA tests provide a rapid, inexpensive and safe means of conducting serologic investigations. AAHL initially developed an indirect ELISA for the detection of IgG antibodies to Hendra virus in horses. Subsequently, a range of refinements has been explored to reduce non-specific reactions and improve test specificity relative to the SNT. A competitive ELISA format using monoclonal antibodies is now being developed. The incorporation of protein A and protein G conjugates rather that anti-species conjugates has broadened the application of the indirect ELISA beyond horses (Daniels *et al.*, 2001a). Notwithstanding, there are a number of shortcomings of the present ELISA in relation to Hendra virus surveillance in wildlife populations.

2.6.3.2 Serologic test limitations for wildlife species

Serologic tests specifically developed for diseases of wildlife are limited, and those used in wildlife studies are commonly transposed from domestic species. The validity of such tests and the meaningful interpretation of test results can therefore be problematic. Gardner *et al* (1996) raise two fundamental points on the transposition of serologic tests to wildlife species. They firstly note that many tests have not been adequately evaluated in the domestic species for which they were developed, and thus lack data on inherent test sensitivity and specificity. Secondly, they argue that even if the test has been validated in domestic species, test characteristics should not be assumed to be the same in wildlife species, given possible differences in pathogen strains, host responses, and exposure to cross-reacting infections in the wildlife species. If Se and Sp are unknown, only seroprevalence and not true prevalence can be reported, with the former over- or underestimating true prevalence by an unknown amount. Further, the authors note that in

the absence of test Se and Sp, neither the predictive value of the test, nor population Se and Sp, can be determined.

However, the authors make a number of positive observations. Firstly, as discussed above, many human and domestic animal ELISA assays use species-specific reagents. While such reagents are rarely available for wildlife species, the associated potential major impediment to serologic investigations of wildlife populations has been largely overcome by the development of protein A and/or G complexes which are used as alternatives to secondary antibody in these tests. Secondly, while test sensitivity and specificity can be difficult and expensive to establish, it is possible to obtain relative values by comparison with known positive and negative samples. Thirdly, as ELISA Se and Sp are directly influenced by the cut-off value, it may be appropriate to present results as a ROC curve[3], or report results using several cut-off points. Fourthly, ELISAs using monoclonal antibodies can reduce cross-reactivity between antigenically related agents, and thus improve specificity. Fifthly, the positive predictive value of a test can be improved by testing high-risk groups, or by retesting 'positive' samples and using series interpretation[4] of the results.

The validity of the current Hendra virus ELISA for wildlife populations remains unknown. While the high costs and logistical issues associated with serum neutralisation tests are a major constraint, these are balanced by the high sensitivity and specificity relative to the ELISA, and the absence of possible species-specific complications. Thus the Hendra virus SNT is the preferred serologic test in this research.

2.7 MODELLING DISEASE IN WILDLIFE POPULATIONS

2.7.1 Introduction

Models are simplified representations of real systems or processes. From an epidemiological perspective, mathematical modelling offers a theoretical approach to disease investigation, complementing observational studies and experimental studies, and is a particularly useful in explaining and predicting patterns of infectious disease in populations. Anderson and May (1979) produced a seminal work on infectious disease

[3] The ROC curve illustrates test Se and Sp for all possible cut-off points by plotting the true positive proportion (sensitivity) on the y-axis and the false positive proportion (1-specificity) on the x-axis.

[4] In series interpretation, only those sera that are positive in both tests are interpreted as positive.

modelling using a compartmental model that incorporated susceptible, infected and immune states with the flow of individuals between states controlled by rate parameters. This state-transition approach has been widely adopted in animal studies, and is used in Chapter 6 to investigate the dynamics of Hendra virus infection in flying foxes. State-transition models for microparasitic infections are the primary focus of this review.

2.7.2 State transition models

In introducing infectious disease models, Graat and Frankena (2001) differentiate between microparasitic infections (viral, bacterial and protozoal infections) and macroparasitic infections (for example, gastrointestinal nematodes). In state-transition models for the former, individuals fall into a number of distinguishable states between which they move over time. Animals that are susceptible but not yet infected are classified as susceptible (S); those that are infected and infectious are classed as infectious (I); and those that have died, or have recovered and are completely immune (R)[5]. $S_{(t)}$, $I_{(t)}$, and $R_{(t)}$ are expressed as proportions of the total population (N) at time t. These states are regarded as variables, and the rate of transition between the states, as parameters. Markov chain models are examples of a simple state-transition model with a constant probability of infection. In such models, the number of individuals becoming infected for example, depends solely on the number of susceptible individuals multiplied by a fixed probability ($p_{si}*S_{(t)}$). Other state-transition models have variable transition probabilities, where the transition probability depends on the number of infectious individuals in the previous time period ($I_{(t+1)} = S_{(t)} *(1-Q^{I(t)})$)[6]. The assumption is that infection is by direct contact, and that each an infectious individual has contact with a finite number of individuals, a proportion of which are susceptible. The Reed-Frost model is an example (Graat & Frankena, 2001).

In an SIR model of constant population size[7] (N), the number of individuals flowing per unit time from the susceptible (S) to the infectious (I) state depends on the proportion of infectious individuals ($I_{(t)}$), the proportion of susceptible individuals ($S_{(t)}$), and the rate of infection (b). That is

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[5] Hence these models are commonly called SIR models.

[6] Q is the probability of avoiding effective contact, and equals 1-P, where P is the probability of effective contact.

[7] Open population SIR models can also be developed to incorporate immigration/births/purchases and emigration/deaths/culling.

$b * S_{(t)} * I_{(t)} * N$.

Thus, the proportion of susceptible individuals over time is

$$dS_{(t)}/dt = b * S_{(t)} * I_{(t)} * N/N$$
$$= b * S_{(t)} * I_{(t)}.$$

Similarly, the proportion of removed individuals over time is

$$dR_{(t)}/dt = a * I_{(t)} * N/N$$
$$= a * I_{(t)}$$

where *a* is the rate at which infectious individuals are removed.

The proportion of infectious individuals over time is the difference:

$$dI/dt = b * S_{(t)} * I_{(t)} - a * I_{(t)}$$

The derivation of these formulae reflects the approach used in chemical reaction kinetics and assumes mass-action theory, wherein interaction between particles is random. Bouma *et al* (1995) demonstrated the assumption to be valid in randomly mixing animal populations (Graat & Frankena, 2001).

The basic reproductive number R_0 is an important concept in SIR models. It represents the average number of new cases that arise from one infectious individual, and equals *b/a*. If R_0 is greater than 1, the probability of a major outbreak is increased; if less than 1, the outbreak is likely to be limited.

2.7.3 Challenges in wildlife modelling

While modelling disease in wildlife populations encounters many of the same challenges common to modelling in other populations, some issues are of particular relevance to wildlife. These generally stem from the uncontrolled nature of wildlife populations, and not infrequently from an incomplete knowledge of the basic biology of many wildlife species. A number of pertinent issues are reviewed below.

2.7.3.1 The mode of transmission

An understanding of the mode of transmission is fundamental to robust modelling, but is frequently limited or lacking in wildlife diseases. Most models of host-pathogen systems assume transmission by so-called ‘mass action’ as discussed above, however there has been some debate over whether mass action models should correctly use numbers or densities of susceptible and infected hosts (Bouma *et al.*, 1995; de Jong, 1995; McCallum *et al.*, 2001). De Jong *et al* (1995) argue for the latter, contending that where densities

remain constant, transmission is dependent of population size. McCallum *et al* (2001) contend that the issue only arises when population size is dynamic, and suggest that a scenario where numbers vary but density remains constant is unusual in wild populations. They contend that the rate of transmission in such populations depends on the number of individuals with which a particular individual is likely to interact, rather than the total number of individuals in the population. This contention reflects that of Barlow (1991) (de Jong, 1995), who in a study of bovine tuberculosis(*Mycobacterium bovis*) in possums, observed that infection was patchy in space, with infected hosts more likely to have infected neighbours than would occur with random mixing. McCallum *et al* (2001) note likely exceptions to the 'neighbourhood' model, exampling disease transmission between seals in discrete compact colonies, and suggesting that in such scenarios it is reasonable to treat the entire colony as the neighbourhood. However, importantly, in their concluding remarks, they recommend evaluation of several alternative transmission models against observed disease patterns wherever possible. They further recommend a clear statement and justification of the form of transmission used in disease models, including whether S, I and N are numbers or densities.

2.7.3.2 Heterogenous mixing and non-linear transmission

Barlow (2000), in discussing improved modelling of bovine tuberculosis in possums, contends that there are two main arguments for assuming heterogenous-mixing and non-linear transmission, and that these may well apply to other wildlife disease systems. The first is that infection in the natural (possum) host was spatially patchy. This he argues, most likely reflects local heterogeneity in possum density (a function of carrying capacity), and that patches of disease likely occur where host density is above the threshold for persistence. These patches, he contends, are separated by areas of sub-threshold host density, and this spatial mosaic is overlain with a dynamic 'pattern' of stochastic extinctions and re-infections. Barlow's second argument is that the existing homogenous-mixing models appear unable to produce the main features of the observed pattern of the disease. He cites the inability of the homogenous-mixing model to simultaneously give a low host threshold for disease elimination *and* a high equilibrium density in the presence of disease (as occurs in nature). The heterogenous-mixing model does achieve this, and he further argues, yields a more stable disease pattern with a relatively rapid recovery after a single reduction in host density, whereas homogenous-mixing models tend to give an unrealistic oscillatory pattern. He concludes that main reason for the generally more realistic behaviour of the heterogenous-mixing model is that

for the same (realistic) equilibrium disease prevalence and host density, it allows a higher value for the transmission coefficient than does the homogenous-mixing model.

McCallum *et al* (2001) suggest that physiological heterogeneity in susceptibility may be responsible for a non-linear relationship between time and the number of new infections, with highly susceptible individuals rapidly becoming infected, and less susceptible individuals becoming infected later at a slower rate. This argument is intuitive and biologically sound.

2.7.3.3 Model parameterization

Barlow (2000) emphasizes the importance of realistic parameter values on realistic model outcomes. While acknowledging the value of simple disease-host models to illustrate the qualitative (data-free) behaviour of the disease-host relationship, he argues that the ability to draw robust semi-quantitative conclusions on the dynamic and control of disease requires more attention to parameter detail. He contends that as models frequently have several parameters based on poor or non-existent data, it is important that realistic values be used for those that can be quantified.

2.8 CHAPTER CONCLUSIONS

The emergence of these viruses, the description of their clinical manifestations, and the findings of the experimental studies represent an intriguing chapter in the annals of animal and public health in Australia. However, while substantial progress has been made in understanding the pathogenesis of both agents, little has been forthcoming about the ecology of Hendra virus and Australian bat lyssavirus. While much is known about the biology of flying foxes in Australia, detailed knowledge of population movement dynamics is lacking. Similarly limited are diagnostic capabilities specific for flying foxes. The following chapters describe the epidemiological investigations in relation to the identification of the putative natural hosts of both agents, the distribution and frequency of both agents in Australia, aspects of the ecology of the viruses in their natural host, and a predictive model illustrating HeV infection dynamics in a flying fox population.

CHAPTER THREE

INVESTIGATIONS OF THE ORIGIN OF HENDRA VIRUS
– a cross-sectional study series

3.1 Introduction

Hendra virus was first described in 1994 in Australia after a sudden outbreak of an acute respiratory syndrome in thoroughbred horses in a Brisbane training stable (Murray *et al.*, 1995b). The putative index case in this outbreak was the heavily pregnant mare Drama Series, initially at pasture in the Brisbane suburb of Cannon Hill. When observed to be ill, she was moved to the Hendra stable for nursing, but died after a 2-day illness. A further twelve horses in the stable and an adjoining training stable died acutely over the subsequent fourteen days. The situation was compounded when the trainer and a stable hand, both directly involved in nursing the index case, contracted a severe febrile illness within a week of contacting the index case. The trainer was hospitalised and died after a brief illness. Hendra virus had emerged. Hypotheses as to the origin of the virus included contaminated biological products, illegal performance-enhancing substances and malicious intent. When investigations failed to support any of these scenarios, consideration was given to the possibility of a wildlife reservoir. This chapter describes the surveillance of wildlife that followed.

The investigations are presented as three successive studies to best represent the sequence of events: preliminary wildlife surveillance, investigations of the role of flying foxes, and investigation of other wildlife species.

3.2 PRELIMINARY WILDLIFE SURVEILLANCE: INVESTIGATIONS OF THE ORIGIN OF HENDRA VIRUS

3.2.1 Introduction

The emergence of Hendra virus caused consternation for animal and public health authorities alike. Zoonotic infections of horses were previously unknown, yet it was evident that the death of the trainer was attributable to his close contact with the index horse case. When the aetiological agent was established as a novel virus of the family

Paramyxoviridae (Murray *et al.*, 1995b), the search for its origin began. To evaluate the hypothesis that the virus existed in a wildlife reservoir, a preliminary serological survey of wildlife species was undertaken. The focus was initially the index case paddock at Cannon Hill, but was later broadened to include a second property in central Queensland when an earlier outbreak on the property was retrospectively identified. The primary objective was to screen wild-caught wildlife from both properties for evidence of Hendra virus infection.

3.2.2 Materials And Methods

3.2.2.1 Study design

Surveillance was conducted using a cross-sectional study design at the individual animal level. The study population comprised animals from non-randomly (convenience) selected populations in Queensland.

3.2.2.2 Sampling locations

Brisbane index case paddock

The study area was the 70-hectare index case property in the Brisbane suburb of Cannon Hill. The aspect of the paddock was southern, with a gentle slope from north to south. Paddock vegetation was predominantly degraded pasture (*Cynodon dactylon*, *Kikuyu* sp.), with an open woodland structure of *Eucalypt* spp. and *Acacia* spp. in the northeastern portion of the paddock. There were two avenues of native fig trees (*Ficus* spp.) in the southern portion of the paddock. Major earthworks associated with a housing development were underway in the northwestern quarter of the paddock. Historically, the area was an abattoir holding-paddock and stockyard complex. Major arterial roads bounded the northern and eastern sides, and a suburban rail line the southern side. Land-use on the eastern and western sides was suburban housing, and on the northern and southern sides, light industry and commercial premises. Horses were agisted in the southern portion of the paddock, an area of approximately 20 hectares. Sampling was concentrated in, but not confined to, this area.

Mackay index case paddock

The study area was the 66-hectare index case property in a rural area approximately 40 kilometres north of Mackay in central Queensland. Approximately 30 hectares was cultivated for sugar-cane production, with the balance providing a mix of native and improved pasture grazing paddocks for a thoroughbred horse stud. The property, situated in a valley running north-south, was bounded by a road on its eastern side (with a cane farm opposite), cane

farms on the northern and southern sides, and remnant and regrowth closed forest on its western side. A semi-permanent creek, lined in part with mature *Melaleuca* sp. trees, ran through the grazing paddocks.

Multiple central Queensland locations

Eleven rural and urban locations in the Mackay and Rockhampton localities constituted the study areas. The locations were the residential properties of selected volunteer wildlife rehabilitators who had a range of sick and injured free-living wildlife in temporary captivity.

3.2.2.3 Sampling methodology

Brisbane index case paddock

Live-catch cage traps of three sizes were employed: Size A Elliott™ traps (a collapsible aluminium 'box' trap 80x80x300mm, with a treadle -activated trigger releasing a spring-loaded hinged door); rat traps (a wire mesh cage trap 150x150x400mm, with a baited hook-activated trigger releasing a hinged door); and possum traps (a wire mesh trap 350x350x650mm, with a treadle-activated trigger releasing a falling sliding door). The Elliott™ and rat traps were baited with either a stiff mixture of peanut paste and rolled oats, or a commercial dry dog food; the possum traps were baited either with a commercial tinned fish cat food or a grain and molasses mix. Feeding stations were sometimes established in the weeks preceding trapping to attract wildlife to proposed trap locations. The traps were positioned in locations judged to provide food or shelter for native wildlife species or introduced feral and pest species, or at locations identified by visitations to feeding stations, or by trapping success rate. Locations commonly included creek lines, patches of remnant vegetation, grassed headlands in cane paddocks, and farm buildings.

Mackay index case paddock

Sampling methodology was the same in Mackay as in Brisbane.

Multiple central Queensland locations

Contact was made with Queensland Parks and Wildlife Services and with volunteer wildlife carer groups in Mackay and Rockhampton, and a list of local wildlife carers was compiled. Species that were common to the Brisbane and the Mackay index case paddocks, and particularly those species that were able to move readily between the two locations, were prioritised for screening.

3.2.2.4 Sample size

Minimum sample size targets were consistent for the three surveys. A minimum sample size per species of 10 was sought, enabling infection to be detected (with 95% statistical confidence) where the population prevalence was 25% or higher, assuming 100% test sensitivity and specificity. The minimum sample size was not always obtained.

3.2.2.5 Sampling period

Brisbane index case property

Three trapping visits were made in the month of May 1995, the night of May 18 being the primary trapping effort. The nights of June 1 and June 20 were follow-up efforts directed primarily at species known to be present but not yet successfully captured.

Mackay index case property

Sixteen trapping efforts were undertaken, over a period of 18 days, between November 1 and November 8, and November 29 and December 8, 1995.

Multiple central Queensland locations

Cooperating wildlife carers in Mackay and Rockhampton were visited on at least one of two occasions. In Mackay, the first occasion was at the time of the second trapping period at the Mackay index case paddock (November 29 and December 8, 1995), and the second occasion was between April 19 and April 20, 1996. In Rockhampton, the first occasion was between April 1 and April 2, 1996 and the second, on April 18, 1996.

3.2.2.6 Specimens collection

Brisbane index case paddock

Captured animals were held for up to one hour, either in the trap, or in Hessian bags, depending on species. Sample collection involved sedation, anaesthesia, or euthanasia, depending on species, and collection of a blood sample by venipuncture or cardiac puncture (again depending on species). Blood was either collected into a plain tube (with or without a heparinized syringe), or into a heparinized tube, depending on species. Samples were left to stand at room temperature overnight, serum or plasma harvested and stored under refrigeration, and forwarded to YVL. Additionally, fresh tissue samples of liver, lung, spleen, kidney, and large bowel were collected from rodents for virus isolation attempts.

Mackay index case paddock

Sample collection procedure in Mackay was the same as that described for Brisbane.

Multiple central Queensland locations

Sample collection involved physical or chemical restraint, depending on species, and collection of a blood sample by venipuncture of a peripheral vein. Blood was collected into a plain tube with a heparinized syringe. Samples were handled as described above.

3.2.2.7 Laboratory methodologies

Mammalian sera were screened for antibodies to Hendra virus at YVL using an indirect ELISA test incorporating inactivated Hendra virus antigen and a protein G-HRP conjugate. (Protein G-HRP conjugate was used in the absence of an anti-species antibody conjugate.) ELISA-positive sera were forwarded to AAHL for confirmation by SNT. All avian, amphibian and reptile sera were forwarded directly to AAHL for testing by SNT[8]. Ectoparasites were forwarded directly to AAHL for antigen screening by PCR.

3.2.2.8 Data management and analysis

All data were stored and managed in a Microsoft ™ Access 97 ™ database. Data was exported to Microsoft ™ Excel 97 ™ for descriptive analyses.

3.2.3 Results

Brisbane index case property

A total of 26 samples from 5 species were tested (Table 3.1). All samples tested negative for antibodies to Hendra virus.

Mackay index case property

A total of 142 samples from at least 16 species were tested (Table 3.2). All samples tested negative.

[8] The validity of using a protein G-HRP conjugate in the absence of anti-species antibody conjugates has not been demonstrated for non-mammalian species.

Table 3.1: Characteristics of 26 wild-caught native, feral and pest species surveyed on the Brisbane index property in May 1995.

Species	Number of animals	
	Submitted	Testing positive by ELISA
House mouse (*Mus musculus*)	19	0
Northern brown bandicoot (*Isodoon macrourus*)	3	0
Black rat (*Rattus rattus*)	2	0
Brown rat (*Rattus norvegicus*)	1	0
Feral domestic cat (*Felis domesticus*)	1	0
Total	26	0

Table 3.2: Characteristics of 142 wild-caught native, feral and pest species surveyed on the Mackay index property in November and December 1995.

Species	Number of animals	
	Submitted	Testing positive by ELISA
Cane toad (*Bufo marinus*)	49	0
Northern brown bandicoot (*Isodoon macrourus)*	27	0
Pooled ectoparasites from *I. macrourus*	13	0
Canefield rat (*Rattus sordidus)*	13	0
Black rat (*Rattus rattus)*	7	0
House mouse (*Mus musculus)*	5	0
Grassland Melomys (*Melomys burtoni*)	5	0
Domestic turkey (*Meleagris sp.)*	5	0
Guinea fowl	5	0
Brush-tailed possum (*Trichosurus vulpecula)*	4	0
Peacock (*Pavo cristatus*)	3	0
Fawn-footed Melomys (*Melomys cervinipes*)	2	0
Eastern Brown snake (*Pseudonja textilis)*	1	0
Skink (*Carlia* sp.)	1	0
Coucal pheasant (*Centropus phasianinus)*	1	0
Domestic chicken (*Gallus domesticus)*	1	0
Total	142	0

Multiple central Queensland locations

A total of 66 samples from at least 15 species were tested (Table 3.3). Sera of six of 21 *P. alecto* and five of six *P. conspicillatus* were positive by ELISA. All ELISA-positive sera were positive for neutralising antibodies to Hendra virus by serum neutralisation test. All other samples were negative for antibody or antigen.

Table 3.3: Characteristics of 66 native species in temporary captivity surveyed in Mackay (December 1995 and April 1996) and Rockhampton (April 1996).

Species	Number (%) of animals	
	Submitted	Testing positive by ELISA and SNT
Black flying-fox *(Pteropus alecto)*	21	6 (29%)
Agile wallaby (*Macropus agilis*)	8	0
Pooled ectoparasites from *Pteropus alecto*	7	0
Spectacled flying-fox *(Pteropus conspicillatus)*	6	5 (83%)
Grassland whistling duck *(Dendrocygna eytoni)*	6	0
Pacific black duck *(Anas supersiliosa)*	6	0
Whiptail wallaby (*Macropus parryi*)	3	0
Owlet nightjar (*Aegotheles cristatus*)	2	0
Frill-necked lizard (*Chlamydosaurus kingii*)	1	0
Australian magpie (*Gymnorhina tibicen*)	1	0
Magpie lark (*Grallina cyanoleuca*)	1	0
Pooled ectoparasites from *Wallabia bicolor*	1	0
Swamp wallaby (*Wallabia bicolor*)	1	0
Pooled ectoparasites from *Macropus parryi*	1	0
Rufous bettong (*Aepyprymnus nufescens*)	1	0
Total	66	11

3.2.4 Discussion

Initial surveillance of wildlife focused on the Brisbane index case paddock. Rodents were targeted for surveillance because Campbell *et al* (1977) had previously isolated an undescribed virus of the family *Paramyxoviridae* from species of rodent in Queensland. While no evidence of infection was found in sampled wildlife in the index case paddock, the small number of species sampled and the small sample size per species limited meaningful interpretation of the data. To a lesser extent, the same limitations were encountered in the survey of the Mackay index case paddock, notwithstanding the use of a larger range of trap sizes and bait types, and pre-trapping feeding regimes. In addition to the limited species diversity and varying population densities in both index case paddocks,

the limited species representation and sample size also reflects the limited scale of trapping and the limitations of trapping as a sampling methodology *per se*. Interpreted more broadly, the modest capture success reflects some of the problems inherent in surveying uncontrolled populations, and prompted consideration of the targeted approach to subsequent wildlife surveillance discussed below.

Temporal considerations also compounded the difficulty in interpreting the negative findings of the index paddock surveys. The Brisbane and Mackay paddock surveys were undertaken nine and 15 months respectively after the equine index cases. For surveyed species with a short generational length, such as rodents, a time lag of this magnitude would allow a substantial population turnover. Additionally, the seasonal disparity between the survey and the index case in both locations further compounded interpretation of the negative results.

When surveillance of the Brisbane and Mackay index case paddocks failed to find evidence of HeV infection in wildlife, the sample base was broadened to include sick and injured wildlife in temporary captivity. Apart from broadening the sample base in terms of species and location, these animals offered the additional advantages of a convenient and cost-effective sample, and access to that subset of the greater wildlife population in sub-optimal health. Access to the latter was of particular interest, because if HeV infection in flying foxes was associated with clinical or sub-clinical disease, then infected animals would be over-represented in this group. Targeted wildlife species in care were sampled in Mackay, and later in Rockhampton, several hundred kilometres to the south of Mackay. The choice of Rockhampton was primarily logistical. The determination of target species reflected two events: the findings of a study which indicated an absence of nucleotide sequence variation in HeV isolates from Mackay and Brisbane, locations 800 km apart (Hooper *et al.*, 1996); and the outcome of a multidisciplinary 'think tank' convened by DPI&F in January 1996, which prioritised species that were common to the two locations and able to move readily between the two locations (Young *et al.*, 1996). Flying foxes were the only mammalian species meeting these criteria. The knowledge that viruses of the family *Paramyxoviridae* had previously been isolated from bats elsewhere (Henderson *et al.*, 1995; Pavri *et al.*, 1971) reinforced the 'think tank' conclusion. Serosurveillance of flying foxes in care in Rockhampton immediately identified neutralising antibodies to HeV in *P. alecto*. Further sampling of this species in Mackay, and of *P. conspicillatus* in Rockhampton, identified additional seropositive individuals. Of the 27 flying foxes tested,

40% were positive by SNT. However, given the opportunistic sampling methodology and the consequent non-representative nature of the sample, and the small sample size, it is inappropriate to base a population estimate on this sample. Moreover, if antibody-positive flying foxes have a greater probability of coming into care, this figure will overstate the true prevalence by an unknown amount.

Notwithstanding the limitations of this preliminary study, the finding of neutralising antibodies to Hendra virus in flying foxes was a major breakthrough in the search for the origin of Hendra virus. The next section describes the subsequent investigation of the role of flying foxes in the ecology of Hendra virus.

3.3 INVESTIGATIONS OF THE ROLE OF FLYING FOXES (*CHIROPTERA*; *PTEROPODIDAE*) IN THE ECOLOGY OF HENDRA VIRUS.

3.3.1 Introduction

The preliminary wildlife surveillance undertaken after the Brisbane and Mackay outbreaks sought to test the hypothesis that Hendra virus existed in a wildlife reservoir. When neutralising antibodies to Hendra virus were discovered in 40% of a non-random sample of 27 flying foxes, the DPI&F undertook an integrated program of research into the role of flying foxes in the ecology of Hendra virus. The program incorporated concurrent virological, pathogenesis, and epidemiological studies. This section presents the epidemiological study.

The primary objectives were to

1. Describe the spatial distribution of anti-Hendra virus antibodies in flying fox populations across their mainland distribution (Study 1);
2. Determine whether Hendra virus infection in flying foxes was recent or historic (Study 2);
3. Identify risk factors for infection in flying foxes (Study 3).

3.3.2 Materials and Methods

3.3.2.1 Study design

Surveillance was conducted using a cross-sectional study design at the individual animal level. The study population comprised flying foxes from non-randomly (convenience) selected populations on mainland Australia.

3.3.2.2 Sampling locations

Studies 1&3

Flying foxes were non-randomly sampled by one of two methodologies: wild-caught or opportunistic. Wild-caught samples were collected from localities in southeast Queensland (Brisbane, Ipswich, Esk, Munduberra) north Queensland (Townsville), New South Wales (Sydney), the Northern Territory (Shady Creek), and Western Australia (Kununurra, Broome, Millstream). Each locality had at least one apparently permanent flying fox camp, although the number of flying foxes in residence varied seasonally. Some localities had multiple capture sites. Typical of flying-fox camps, all surveyed camps were in the riparian zone, and (with the exception of the Northern Territory), roost trees were either predominantly mangrove species (Indooroopilly Island and Norman Creek in Brisbane, Ross River in Townsville, and Broome) or eucalypts or melaleucas (Ipswich, Esk, Mundubbera, Kununurra, and Ku-ring-ai and Cabramatta in Sydney). The surveyed Northern Territory colony was roosting in a large stand of (exotic) bamboo. The camps were often in or near urban areas (Indooroopilly Island and Norman Creek in Brisbane, Esk, Ross River in Townsville, Woodend in Ipswich, Cabramatta in Sydney, and Kununurra and Broome). Opportunistic samples were obtained from wildlife authorities, carers, and institutions in Lismore and Gordon (Sydney) in New South Wales, and from Ipswich, Brisbane, the Gold Coast, Wide Bay and Millaa Millaa in Queensland. No samples were collected from Victoria for logistical reasons, and because the small numbers of flying foxes in that state are believed to be an extension of the NSW populations. Flying foxes are not found in Tasmania or South Australia (Figure 3.1).

Study 2

Archived sera were obtained from two sources. CSIRO (Long Pocket, Brisbane) had sera from 12 *P. scapulatus* that were collected in 1976 from a grazing property (Bow Park) on the Saxby River, 60 km north of Julia Creek in north Queensland. The University of Queensland Department of Physiology and Pharmacology made available 68 sera from

several flying fox species collected from several locations in southeast Queensland between 1982 and 1984.

Figure 3.1: Distribution of flying foxes on mainland Australia[1] and sampling locations for Study 1.

Key: Horizontal hatching = *P. alecto*
Vertical hatching = *P. poliocephalus*
Solid black = *P. conspicillatus*
Broken line = southern inland limit of *P. scapulatus*.

[1] Adapted from Hall and Richards (2000).

3.3.2.3 Sampling methodology

Studies 1 & 3

As previously stated, flying foxes were non-randomly sampled by one of two methodologies: wild-caught or opportunistic. Wild-caught specimens were collected by mist-net (Box 3.1) (Queensland, Northern Territory) and by shooting (Western Australia). Shooting was used where mist-netting was not feasible, and was undertaken by a trained and authorised shooter. Opportunistically sampled flying foxes came from two sources. Either they were diagnostic specimens found sick, injured or recently dead, and submitted to YVL for Hendra virus or ABLV exclusion, or they were temporarily captive sick or injured animals being rehabilitated by wildlife carers (Queensland, New South Wales).

Box 3.1: Sampling free-living flying foxes by mist-net.

A mist-net (20m long and 3m deep, with a synthetic polyfilament 1mm thread forming a 50x50mm mesh) was suspended between two nine-metre high aluminium masts located in an observed flight path and within 20 meters of the edge of the flying fox camp. Using a simple pulley system, two operators (one at the base of each pole) working in unison could raise and lower the net within five seconds. Animals were caught as they left the camp at dusk, or as they returned to camp in the hours before dawn. The net was immediately lowered when a flying fox became caught in it, the animal removed before it became heavily entangled, placed in a hanging bag, and the net re-hoist. Animals were held for a maximum of four hours.

Study 2

The sampling methodology used to obtain the archived samples is unknown. All samples from both archives were screened.

3.3.2.4 Sample size

Minimum sample size targets were consistent for the three studies. A minimum sample size of 30 individuals per species was sought, enabling detection of antibodies (with 95% statistical confidence) at a minimum population seroprevalence of 10%, assuming 100% test sensitivity and specificity. The minimum sample size was not always obtained.

3.3.2.5 Sampling period

Studies 1 & 3

The sampling period in Queensland was April 1996 to December 1998; in New South Wales, April 1996 to February 1998; in the Northern Territory, August 1999; and in Western Australia, December 1998.

Study 2

The sampling periods were 1976, and 1982 to 1984.

3.3.2.6 Specimen collection

Studies 1 & 3

Wild-caught flying foxes were typically bled and released. If tissue samples were sought for Hendra virus isolation attempts, or for a concurrent survey of ABLV, flying foxes were euthanased by lethal injection (Lethabarb™) and brain, lung, liver, kidney, spleen, testes or uterus collected. Diagnostic specimens were necropsied and the same range of tissues collected. Rehabilitating animals in temporary captivity were bled and returned to care. Blood was collected from live flying foxes by venipuncture and from euthanased flying foxes by cardiac puncture.

Study 2

The collection methodology of the archived sera is unknown.

3.3.2.7 Laboratory methodologies

Sera were screened for antibodies to Hendra virus at YVL using an indirect ELISA test incorporating Hendra virus antigen and a protein G HRP conjugate. Sera testing positive by ELISA were forwarded to AAHL for confirmation by SNT.

3.3.2.8 Data management and statistical analysis

Data were recorded on six variables: *Species*, *Sex*, *Age*, *SampleLocation*, *SampleYear*, *SampleMethod*, and *HeV Serostatus*. *Age* (immature, mature) was based on morphometric and physiological criteria indicating sexual maturity (attained by the majority of animals at 30 months) (Hall & Richards, 2000; McIllwee & Martin, 2002), and included weight and forearm length, dentition wear, and mammary development. All data were stored and managed in a Microsoft™ Access 97™ database. Data was exported to Microsoft™ Excel 97™ for descriptive and univariate analyses, and to Stata™[9] for multivariate analysis.

Univariate analyses employed a chi square test to measure associations between the outcome variable *HeV Serostatus* (excluding entries with unknown serostatus[10]) and explanatory variables, with *p* values indicating the statistical significance of associations and unadjusted odds ratios indicating the magnitude of associations[11]. Yates Corrected *p* value was used except where cell values were less than 5, when Fisher's Exact *p* value was used.

The multivariate analysis in Study 3 utilised a logistic regression approach. To facilitate a comprehensive analysis, the dataset for multivariate analysis excluded entries with unknown age, sex and HeV serostatus. In addition, sparseness of data in several fields was overcome by collapsing *SampleLocation* fields into WA+NT and Qld+NSW, and *SampleYear* fields into 96/97 and 98/99. Coding was 0/1 for *HeV Serostatus* (negative/positive), *Age* (immature/mature), *Sex* (male/female), *SampleMethod* (wild-caught/opportunistic), *SampleLocation* (WA+NT/Qld+NSW), and *SampleYear* (96+97/98+99). For *Species*, dummy variables were created (*P. poliocephalus*=1, *P. alecto*=2, *P. conspicillatus*=3), with *P. scapulatus* the referent species. The coded dataset was exported to Stata™. An unweighted fixed-effects logistic regression model with *HeV Serostatus* as the outcome variable was manually constructed by means of a deviance table. Because of the potential importance of an association with *HeV serostatus*, the explanatory variables *SampleYear* and *SampleLocation* were forced into the model regardless of statistical significance. Inclusion of the remaining variables was dependent on their contributing a significant

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[9] Stata 8.2 for Windows™, Stata Corporation, College Station, Texas, USA.

[10] Entries with an unknown serostatus were analysed separately to identify any significant associations with this outcome.

[11] Unadjusted odds ratios were used in this study (rather than relative risk) to facilitate multivariate analysis using logistic regression.

change ($p<0.05$) in the residual deviance. Interaction terms for these remaining variables were similarly treated. The *p* values for the main effects and interaction terms were based on the likelihood ratio test.

3.3.3 Results

Study 1

A total of 1424 flying foxes representing the four mainland Australian species were obtained from locations in Queensland (8), New South Wales (2), the Northern Territory (1) and Western Australia (3) over a three-year period (Table 3.4). Toxic reaction precluded ascertainment of the serostatus of 252 samples (18%) at all dilutions, leaving 1172 samples with a known HeV serostatus. Of these, a total of 548 sera (47%) tested positive for neutralising antibodies to Hendra virus. Seropositive samples were detected in all four species, and in every surveyed location in every state.

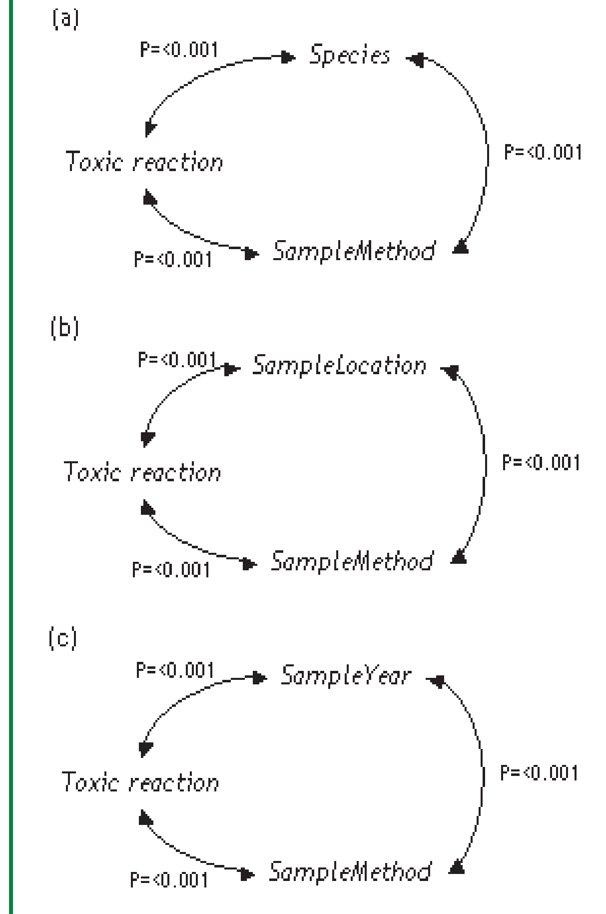
Table 3.4: Characteristics of 1424 non-randomly sampled flying foxes surveyed in Queensland, New South Wales, the Northern Territory and Western Australia between April 1996 and August 1999.

Variable	Number of flying foxes			
	Tested by SNT	Seropositive[1] by SNT	Seronegative[2] by SNT	Unknown serostatus[3]
Species				
P. scapulatus	190	22	140	28
P. poliocephalus	368	133	193	42
P. alecto	697	343	242	112
P. conspicillatus	169	50	49	70
Sex				
Male	748	289	335	124
Female	616	234	270	112
Unknown	60	25	19	16
Age				
Immature	681	243	323	115
Mature	703	294	280	129
Unknown	40	11	21	8
SampleMethod				
Wild-caught	656	294	325	37
Opportunistic	768	254	299	215
SampleLocation				
NT	48	15	33	0
Qld (Southeast)	867	357	367	143
Qld (North)	302	122	89	91
WA (Kununurra)	39	12	27	0
WA (Broome)	37	14	23	0
WA (Millstream)	41	9	32	0
NSW (Lismore)	10	0	3	7
NSW (Sydney)	80	19	50	11
SampleYear				
1996	173	40	80	53
1997	593	207	245	141
1998	610	286	266	58
1999	48	15	33	0
Total	1424	548	624	252

[1] neutralising antibody titre equal to or greater than 1:5
[2] neutralising antibody titre less than 1:5
[3] antibody status was classed as ‘unknown’ where sera produced a toxic reaction in the cell culture at lower dilutions, preventing identification of any neutralisation at these dilutions.

Univariate analysis of the association between the explanatory variables and toxic reaction status identified *Species*, *SampleMethod*, *SampleLocation* and *SampleYear* as significant ($p<0.05$) (Table 3.5). However, a series of additional univariate analyses showed *SampleMethod* was confounding the association of *Species* (Table 3.6), *SampleLocation* (Table 3.7) and *SampleYear* (Table 3.8) with toxic reaction status (See also Box 3.2).

Box 3.2: *SampleMethod* confounded the association of *Species, SampleLocation* and *SampleYear* with toxic reaction status



Study 2

Neutralising antibodies to Hendra virus were found in 16 of 68 sera (24%) and all three identified species represented in the 1982-1984 University of Queensland archive. None of the 12 *P. scapulatus* sera collected in 1976 by CSIRO tested positive (Table 3.9).

Study 3

Univariate analyses of associations between the explanatory variables and *HeV Serostatus* in the 1172 samples of known HeV serostatus identified *Species*, *Age*, *SampleLocation* and *SampleYear* as significant ($p<0.05$) (Table 3.10). These variables remained significant when the analysis was repeated on 1118 samples of known *Age*, *Sex* and *HeV Serostatus* (Tables 3.11 & 3.12). Multivariate analysis of this reduced dataset (Table 3.12) using logistic regression yielded the following model:

> *Constant + Species* (p<0.001) *+ Age* (p=0.006) *+ SampleMethod* (p=0.002) *+ SampleLocation* (p<0.001) *+ SampleYear* (p=0.012)

In addition, the following interaction terms were fitted:

> *Age*Species* (p<0.001) *+ Age*SampleMethod* (p<0.001)

Coeffecients for the main effects and interaction terms are presented in Figure 3.13. Odds ratios for factors involved in interaction terms are presented in Table 3.14. For *SampleLocation* and *SampleYear* (not involved in interaction terms), the odds ratios were 4.35 (95% CI 2.71, 6.99) and 1.53 (95% CI 1.10, 2.12) respectively.

Table 3.5: Univariate association between a number of independent variables and toxic reaction in 1424 non-randomly sampled flying foxes surveyed in Queensland, New South Wales, the Northern Territory and Western Australia between April 1996 and August 1999.

Variable	Number of sera		P value[1]	Unadjusted odds ratio (95%CI)
	Exhibiting toxic reaction	Not exhibiting toxic reaction		
Species			<0.001	
P. poliocephalus	42	326		Referent
P. alecto	112	585		1.49 (1.00, 2.21)
P. conspicillatus	70	99		5.49 (3.44, 8.77)
P. scapulatus	28	162		1.34 (0.78, 2.31)
Sex			0.13	
Male	124	624		Referent
Female	112	504		1.12 (0.84, 1.5)
Unknown	16	44		1.83 (0.96, 3.47)
Age			0.7	
Immature	115	566		Referent
Mature	129	575		1.1 (0.83, 1.47)
Unknown	8	31		1.27 (0.52, 2.98)
SampleMethod			<0.001	
Wild-caught	37	619		Referent
Opportunistic	215	553		6.5 (4.44, 9.58)
SampleLocation			<0.001	
WA + NT	0	165		
Qld + NSW	252	1007		Unable to be calculated[2]
SampleYear			<0.001	
96+97	194	572		Referent
98+99	58	600		0.29 (0.21, 0.39)
Total	252	1172		

[1] P value of chi square statistic.
[2] The zero values precludes calculation.

Table 3.6: Univariate association between the explanatory variables *SampleMethod* and *Species* in 1424 non-randomly sampled flying foxes[1] surveyed in Queensland, the Northern Territory and Western Australia screened for neutralising antibodies to Hendra virus between April 1996 and August 1999.

	SampleMethod		P value
	Wild-caught	Opportunistic	
Species			<0.001
P. scapulatus	156	34	
P. poliocephalus	173	195	
P. alecto	327	370	
P. conspicillatus	0	169	

[1] Table 3.5 data

Table 3.7: Univariate association between the explanatory variables *SampleMethod* and *SampleLocation* in 1424 non-randomly sampled flying foxes[1] surveyed in Queensland, the Northern Territory and Western Australia screened for neutralising antibodies to Hendra virus between April 1996 and August 1999.

	SampleMethod		P value
	Wild-caught	Opportunistic	
SampleLocation			<0.001
WA + NT	165	0	
QLD + NSW	491	768	

[1] Table 3.5 data

Table 3.8: Univariate association between the explanatory variables *SampleMethod* and *SampleYear* in 1424 non-randomly sampled flying foxes[1] surveyed in Queensland, the Northern Territory and Western Australia screened for neutralising antibodies to Hendra virus between April 1996 and August 1999.

	SampleMethod		P value
	Wild-caught	Opportunistic	
SampleYear			<0.001
96/97	182	584	
98/99	474	184	

[1] Table 3.5 data

Table 3.9: Characteristics of 80 archived non-randomly sampled flying fox sera collected in Queensland in 1976 (Julia Creek) and 1982-1984 (south-east Queensland locations).

Species	Number (%) of flying foxes					
	from 1976			from 1982-84		
	Total	Testing positive[1] by SNT	95% CI	Total	Testing positive[1] by SNT	95% CI
P. alecto				20	1 (5%)	0.1-25%
P. poliocephalus				42	13 (31%)	18-47%
P. scapulatus	12	0 (0%)	0-26%	5	2 (40%)	5-85%
Unidentified				1	0 (0%)	0-98%
Total	12	0	0-26%	68	16 (24%)	14-36%

[1] neutralising antibody titre equal to or greater than 1:5

Table 3.10: Univariate association between a number of independent variables and HeV serostatus in 1172 non-randomly sampled flying foxes of known HeV serostatus[1] surveyed in Queensland, New South Wales, the Northern Territory and Western Australia between April 1996 and August 1999.

Variable	Number of flying foxes		P value[4]	Unadjusted odds ratio (95%CI)
	Seropositive[2] by SNT	Seronegative[3] by SNT		
Species			<0.001	
P. scapulatus	22	140		1
P. poliocephalus	133	193		4.39 (2.59, 7.48)
P. alecto	343	242		9.02 (5.47, 15.00)
P. conspicillatus	50	49		6.49 (3.43, 12.36)
Sex			0.39	
Male	289	335		1
Female	234	270		1 (0.79, 1.28)
Unknown	25	19		1.53 (0.79, 1.95)
Age			0.007	
Immature	243	323		1
Mature	294	280		1.4 (1.10, 1.77)
Unknown	11	21		0.7 (0.31, 1.55)
SampleMethod			0.59	
Wild-caught	294	325		1
Opportunistic	254	299		0.94 (0.74, 1.19)
SampleLocation			<0.001	
NT	15	33		1
Qld (Southeast)	357	367		2.14 (1.1, 4.21)
Qld (North)	122	89		3.02 (1.48, 6.22)
WA (Kununurra)	12	27		0.98 (0.36, 2.68)
WA (Broome)	14	23		1.34 (0.49, 3.64)
WA (Millstream)	9	32		0.62 (0.21, 1.78)
NSW (Lismore)	0	3		0 (0.00, 5.88)
NSW (Sydney)	20	50		2.64 (0.83, 8.51)
SampleYear			<0.001	
1996	40	80		1
1997	207	245		1.69 (1.09, 2.64)
1998	286	266		2.15 (1.39, 3.33)
1999	15	33		0.91 (0.42, 1.97)
Total	548	624		

[1] excludes 252 sera of unknown HeV neutralising antibody status due to toxic reaction in the cell culture at lower dilutions.
[2] neutralising antibody titre equal to or greater than 1:5
[3] neutralising antibody titre less than 1:5
[4] P value of chi square statistic.

Table 3.11: Characteristics of 1118 non-randomly sampled flying foxes of known age, sex and HeV serostatus[1] surveyed in Queensland, New South Wales, the Northern Territory and Western Australia between April 1996 and August 1999.

Variable	Number of flying foxes		
	Tested by SNT	Seropositive[2] by SNT	Seronegative[3] by SNT
Species			
P. scapulatus	157	22	135
P. poliocephalus	324	132	192
P. alecto	560	323	237
P. conspicillatus	77	43	34
Sex			
Male	618	286	332
Female	500	234	266
Age			
Immature	545	226	319
Mature	573	294	279
SampleMethod			
Wild-caught	614	293	321
Opportunistic	504	227	277
SampleLocation[4]			
WA+NT	164	50	114
Qld+NSW	954	470	484
SampleYear[5]			
96+97	524	222	302
98+99	594	298	296
Total	1118	520	598

[1] excludes 306 samples of unknown age, sex or HeV serostatus.
[2] neutralising antibody titre equal to or greater than 1:5
[3] neutralising antibody titre less than 1:5
[4] SampleLocation was collapsed to facilitate multivariate analysis (Table 3.9).
[5] SampleYear was collapsed to facilitate multivariate analysis (Table 3.9)

Table 3.12: Univariate association between a number of independent variables and HeV serostatus in 1118 non-randomly sampled flying foxes of known age, sex and HeV serostatus[1] from Queensland, New South Wales, the Northern Territory and Western Australia surveyed between April 1996 and August 1999.

Variable	Number of flying foxes		P value[4]	Unadjusted odds ratio (95%CI)
	Seropositive[2] by SNT	Seronegative[3] by SNT		
Species			<0.001	
P. scapulatus	22	135		1
P. poliocephalus	132	192		4.22 (2.49, 7.20)
P. alecto	323	237		8.36 (5.06, 13.94)
P. conspicillatus	43	34		7.76 (3.92, 15.47)
Sex			0.9	
Male	286	332		1
Female	234	266		1.02 (0.80, 1.30)
Age			0.001	
Immature	226	319		1
Mature	294	279		1.49 (1.17, 1.90)
SamplingMethod			0.37	
Wild-caught	293	321		1
Opportunistic	227	277		0.90 (0.70, 1.15)
SampleLocation[5]			<0.001	
WA+NT	50	114		1
Qld+NSW	470	484		2.21 (1.53, 3.21)
SampleYear[6]			0.01	
96+97	222	302		1
98+99	298	296		1.37 (1.07, 1.75)
Total	520	598		

[1] excludes 306 samples of unknown age, sex or HeV serostatus.
[2] neutralising antibody titre equal to or greater than 1:5
[3] neutralising antibody titre less than 1:5
[4] P value of chi square statistic.
[5] SampleLocation was collapsed to facilitate multivariate analysis (Table 3.9).
[6] SampleYear was collapsed to facilitate multivariate analysis (Table 3.9)

Table 3.13: Final logistic regression model for a positive HeV serostatus in 1118 flying foxes from Queensland, New South Wales, Western Australia and the Northern Territory - coefficients for the main effects and interaction terms[1].

Variable	Coefficient	se	P value[3]
Intercept	-3.39	0.419	
Species			
P. scapulatus[2] (0)	0	-	-
P. poliocephalus (1)	0.96	0.420	0.022
P. alecto (2)	2.00	0.410	<0.001
P. conspicillatus (3)	0.39	0.693	0.577
Age			
Immature[2] (0)	0	-	-
Mature (1)	0.88	0.477	0.064
SampleMethod			
Wild-caught[2] (0)	0	-	-
Opportunistic (1)	0.12	0.223	0.577
SampleLocation			
WA+NT[2] (0)	0	-	-
Qld+NSW (1)	1.47	0.242	<0.001
SampleYear			
96+97[2] (0)	0	-	-
98+99 (1)	0.42	0.167	0.011
Age*Species			
Mature**P. poliocephalus*	0.33	0.550	0.544
Mature**P. alecto*	-0.08	0.530	0.883
Mature**P. conspicillatus*	2.86	0.838	0.001
Age*SampleMethod			
Mature*Opportunistic	-1.56	0.307	<0.001

[1] Final model deviance with 1106 degrees of freedom: 1337
[2] Referent class
[3] Wald test for each coefficient

Table 3.14: Final logistic regression model for a positive HeV serostatus in 1118 flying foxes from Queensland, New South Wales, Western Australia and the Northern Territory – derived coefficients for factors involving interaction terms[1].

Variable	Coefficient	se	P value[3]	Odds Ratio	95%CI
Species within *Age* within *SampleMethod*					
Wild-caught					
Immature					
P. scapulatus[2]	0	-	-	1	-
P. poliocephalus	0.96	0.420	0.022	2.61	1.15, 5.94
P. alecto	2.00	0.410	<0.001	7.38	3.30, 16.48
P. conspicillatus	0.39	0.693	0.577	1.47	0.38, 5.73
Mature					
P. scapulatus	0.88	0.476	0.064	2.42	0.95, 6.16
P. poliocephalus	2.17	0.409	<0.001	8.76	3.92, 19.56
P. alecto	2.8	0.405	<0.001	16.44	7.36, 36.73
P. conspicillatus	4.13	0.216	<0.001	62.18	40.40, 95.70
Opportunistic					
Immature					
P. scapulatus[2]	0	-	-	1	-
P. poliocephalus	0.96	0.420	0.022	2.61	1.15, 5.94
P. alecto	2.00	0.410	<0.001	7.38	3.30, 16.48
P. conspicillatus	0.39	0.693	0.577	1.47	0.38, 5.73
Mature					
P. scapulatus	-0.68	0.554	0.219	0.51	0.17, 1.50
P. poliocephalus	0.61	0.476	0.200	1.84	0.72, 4.68
P. alecto	1.24	0.460	0.007	3.46	1.40, 8.52
P. conspicillatus	2.57	0.500	<0.001	13.07	4.90, 34.81

Table 3.14 continued next page

Table 3.14 continued

Age within *SampleMethod* within *Species*					
P. scapulatus					
Wild-caught					
Immature[2]	0	-	-	1	-
Mature	0.88	0.476	0.064	2.42	0.95, 6.16
Opportunistic					
Immature	0.12	0.223	0.591	1.13	0.73, 1.75
Mature	-0.56	0.523	0.285	0.57	0.21, 1.65
P. poliocephalus					
Wild-caught					
Immature[2]	0	-	-	1	-
Mature	1.21	0.281	<0.001	3.35	1.92, 5.75
Opportunistic					
Immature	0.12	0.223	0.591	1.13	0.73, 1.75
Mature	-0.23	0.705	0.745	0.79	0.21, 2.97
P. alecto					
Wild-caught					
Immature[2]	0	-	-	1	-
Mature	0.80	0.249	0.001	2.23	1.37, 3.63
Opportunistic					
Immature	0.12	0.223	0.591	1.13	0.73, 1.75
Mature	-0.64	0.539	0.235	0.53	0.18, 1.52
P. conspicillatus					
Wild-caught					
Immature[2]	0	-	-	1	-
Mature	3.74	0.699	<0.001	42.10	10.70, 165.67
Opportunistic					
Immature	0.12	0.223	0.591	1.13	0.73, 1.75
Mature	2.3	0.130	<0.001	9.97	7.73, 12.87

[1] Final model deviance with 1106 degrees of freedom: 1337
[2] Referent class
[3] Wald test for each coefficient

Univariate analysis of the association between time of year and *HeV serostatus* was undertaken on 724 flying foxes (380 *P. alecto*, 256 *P. poliocephalus*, 86 *P. scapulatus* and 2 *P. conspicillatus*) surveyed in southeast Queensland between April 1996 and November 1998. There was no statistically significant association (p= 0.79) when species were combined (Table 3.15), but when the analysis was restricted by species, significant or near-significant associations were evident. *P. alecto* surveyed in the first, third and fourth quarters of the calendar year were respectively 2.76 (95% CI 1.37 to 5.56), 1.96 (95% CI 0.94 to 4.1) and 2.27 (95% CI 1.05 to 4.93) times more likely to be HeV seropositive than those surveyed in the referent second quarter (Table 3.16). *P. poliocephalus* surveyed in the second quarter were 2.94 (95% CI 1.16 to 7.89) times more likely to be HeV seropositive than those in the referent fourth quarter. There was no significant difference between the first, third and fourth quarters (Table 3.17). *P. scapulatus* surveyed in the second quarter were 27.60 (95% CI 1.64 to 1477.84) times more likely to be HeV seropositive than those in the referent first quarter. There was no significant difference between the first, third and fourth quarters (Table 3.18).

Table 3.15: Univariate association between time of year and HeV serostatus in 724 *P. alecto*, *P. poliocephalus*, *P. scapulatus* and *P. conspicillatus* of known HeV serostatus[1] surveyed in southeast Queensland between April 1996 and November 1998.

Variable	Number of flying foxes testing		P value	Relative Risk (95% CI)
	HeV positive	HeV Negative		
Time of year			0.79	
January-March	121	131		1.01 (0.65, 1.58)
April-June	95	86		1.21 (0.75, 1.94)
July-September	78	81		1.05 (0.65, 1.72)
October-December	63	69		Referent
Total	357	367		

[1] Table 3.10 data

Table 3.16: Univariate association between time of year and HeV serostatus in 380 *P. alecto* of known HeV serostatus surveyed in southeast Queensland between April 1996 and November 1998.

Variable	Number of flying foxes testing		P value	Relative Risk (95% CI)
	HeV positive	HeV Negative		
Time of year			0.02	
January-March	99	49		2.76 (1.37, 5.56)
April-June	22	30		Referent
July-September	59	41		1.96 (0.94, 4.1)
October-December	50	30		2.27 (1.05, 4.93)
Total	230	150		

Table 3.17: Univariate association between time of year and HeV serostatus in 256 *P. poliocephalus* of known HeV serostatus surveyed in southeast Queensland between April 1996 and November 1998.

Variable	Number of flying foxes testing		P value	Relative Risk (95% CI)
	HeV positive	HeV Negative		
Time of year			0.002	
January-March	17	36		1.05 (0.36, 3.19)
April-June	70	53		2.94 (1.16, 7.89)
July-September	18	33		1.21 (0.42, 3.68)
October-December	9	20		Referent
Total	114	142		

Table 3.18: Univariate association between time of year and HeV serostatus in 86 *P. scapulatus* of known HeV serostatus surveyed in southeast Queensland between April 1996 and November 1998.

Variable	Number of flying foxes testing		P value	Relative Risk (95% CI)
	HeV positive	HeV Negative		
Time of year			0.006	
January-March	5	46		Referent
April-June	3	1		27.60 (1.64, 1477.84)
July-September	1	7		1.31 (0.02, 14.63)
October-December	4	19		1.94 (0.34, 10.03)
Total	13	73		

3.3.4 Discussion

Study 1

The finding of neutralising antibodies to Hendra virus in all flying fox species and locations surveyed is consistent with exposure and infection in flying fox populations over their geographic range on mainland Australia. This extensive spatial scale of infection is not unexpected, given that the distribution of flying foxes in Australia can be represented by a succession of overlapping foraging ranges centred on communal roosting sites which are strategically scattered throughout the geographic range of each species. Aggregates of flying foxes periodically move between roosting sites depending on the availability of food resources proximate to each site. Where geographic ranges of different species overlap, camps are commonly shared by species. Effective contact is further facilitated by the periodic large-scale migration of *P. scapulatus* throughout their geographic range, which overlaps the other mainland species (Hall, 1987) (Figure 3.1). Genetic studies of *P. alecto*, *P. poliocephalus* and *P. scapulatus* indicating an absence of genetic distance within each species across their entire Australian range further emphasise the dynamic nature of Australian flying fox populations (Sinclair *et al.*, 1996; Webb & Tidemann, 1996).

The high proportion of flying foxes with anti-HeV antibodies at each sampling location indicates a high probability of infection and a low case fatality rate in wild flying fox populations. These features have not been seen in natural (Baldock *et al.*, 1996) or

experimental (Westbury *et al.*, 1996; Williamson *et al.*, 1998) infections in other species, and, in conjunction with the wide geographic occurrence of antibodies, support the interpretation that Hendra virus infection is an endemic infection in Australian flying foxes. The isolation from naturally infected flying foxes of HeV isolates of identical nucleotide sequence to isolates from naturally infected horses further supports this interpretation (Halpin *et al.*, 2000).

Of the 1424 sera tested, 252 (18%) were uninterpretable at low dilutions because of toxic effects of the sera on the cell culture. The association with the opportunistic sampling methodology can plausibly be explained by the small volume samples or poor quality samples often obtained from flying foxes sampled in this way. Both are factors typically associated with increased rates of toxic reaction in domestic species.

Study 2

Considerable effort was made to locate archived flying fox sera in museums, universities and other research institutions in Queensland and elsewhere in Australia. The CSIRO and UQ samples, while limited in number and age, nonetheless provide a valuable temporal perspective to the history of Hendra virus in Australia. The presence of anti-Hendra virus antibodies in the 1982-1984 UQ samples demonstrates that Hendra virus infection in Australian flying fox populations preceded the first known infections in horses by at least 10 years. Indeed, the evidence of infection in all three species, in a sample collected in southeast Queensland, supports infection being endemic in Australian flying foxes at that time. The absence of antibodies in the earlier CSIRO sample should not be interpreted as an absence of infection in flying foxes in 1976. While the point prevalence is zero, the 95% confidence interval is 0 – 0.26, and indicates that the absence of infection in this sample may be simply due to chance. Given the likely historic infection in Australian flying foxes, why was disease attributable to Hendra virus not seen in horses or humans before 1994? Two plausible explanations are obvious. Either cases did occur, but were undiagnosed or misdiagnosed, or alternatively, the appearance of Hendra virus infections in horses and humans represents a true example of disease emergence. This discussion will be continued in Chapter 7.

Study 3

In addition to the four variables significantly associated with *HeV serostatus* on univariate analysis (*Species, Age, SampleLocation and SampleYear*), the multivariate analysis

identified a fifth variable (*SampleMethod*) and two interaction terms (*Age*Species* and *Age*SampleMethod*). Considering *Species* first, in both wild-caught and opportunistic samples, the odds for seropositivity for immature *P. poliocephalus* and *P. alecto* were respectively 2.61 and 7.38 relative to immature *P. scapulatus.* Similarly, the odds for mature *P. poliocephalus* and *P. alecto* were respectively 3.62 and 6.79 relative to mature *P. scapulatus*. That is, relative to *P. scapulatus,* the odds ratios for seropositivity in *P. poliocephalus* and *P. alecto* were similar in both age classes, and were significantly higher than 1, regardless of sample method. Interpretation of this finding poses a number of interesting questions. Is *P. scapulatus* less likely to be exposed to infection than other species? Is exposure less likely to result in infection and seroconversion in *P. scapulatus*? Do *P. scapulatus* have a higher case fatality rate, with fewer antibody-positive animals surviving infection? The latter would appear less likely, based on an absence of (albeit limited) field (Halpin *et al.*, 2000) and laboratory (Williamson *et al.*, 2000; Williamson *et al.*, 1998) evidence of clinical disease associated with HeV infection in flying foxes. Certainly the biology of *P. scapulatus* differs in a number of respects to that of *P. poliocephalus* and *P. alecto* (and *P. conspicillatus*), and it is plausible that host or environment factors could influence the likelihood of *P. scapulatus* being either exposed or infected. For example, the reproductive cycle of *P. scapulatus* is countercyclical to the other species (Hall & Richards, 2000). Secondly, within communal camps, *P. scapulatus* are less likely to closely intermix with other species (LS Hall, School of Veterinary Science, UQ; pers. comm.). Thirdly, *P. scapulatus* are nectar and pollen feeders rather than frugivorous (Hall & Richards, 2000). It is also plausible that agent-related factors may influence seroprevalence at the species level. For example, species-specific strains of HeV may occur, with varying infectivity. While the absence of variation among isolates recovered from horses, humans and flying foxes does not support this argument, molecular comparisons of isolates from different flying fox species remain to be done.

In contrast to the similar odds of seroposivity for both age classes in *P. poliocephalus* and *P. alecto*, the odds of mature *P. conspicillatus* being seropositive relative to mature *P. scapulatus* were were much greater (25.68) than the odds of immature *P. conspicillatus* relative to immature *P. scapulatus* (1.47). Again, several scenarios could produce this finding: differing rates of exposure, titre persistence or case fatality between immature and mature *P. conspicillatus*. But a biologically plausible explanation of why this difference should be specific to *P. conspicillatus* is elusive, particularly given the largely parallel biology of *P. conspicillatus*, *P. poliocephalus* and *P. alecto*. Certainly the

distribution of *P. conspicillatus* in Australia is very restricted (to far north Queensland), and further, it is argued that the population has been severely impacted in size, structure and dynamics by culling (McIlwee & Martin (2002). While extralimital populations occur in Papua New Guinea, the level of interaction between the two remains to be quantified. However, notwithstanding the above discussion, selection bias cannot be excluded as a possible explanation of the finding, given that this was a non-random sample.

With respect to *Age*, for each species, mature wild-caught flying foxes were more likely to be seropositive than were immature wild-caught flying foxes. This finding is more consistent with horizontal transmission than with vertical transmission, suggesting a scenario in which older animals have had a greater opportunity over time for exposure and infection (Mills & Childs, 1998). Indeed, it is probable that the true magnitude of the association between age and seroprevalence is stronger than identified in this study, because the serologic test did not differentiate between actively or passively acquired antibody. Thus, an unknown proportion of the immature age class testing seropositive may not have been infected but had circulating maternal antibody. While knowledge of the behaviour of maternal antibodies in flying foxes is limited, a longitudinal study following a number of captive flying foxes (Chapter 4) showed a strong association between dam and offspring serostatus, with the antibody titre in pups of seropositive dams persisting for many months. The lack of an accurate (non-destructive) aging technique for flying foxes limited classification in this study to two age classes (mature and immature) and consequently constrained investigation of the importance of this variable. The ability to identify narrower age cohorts would enable calculation of age-specific prevalence rates and an estimate of incidence.

In contrast to the effects of age on the odds of seropositivity in wild-caught flying foxes described above, mature opportunistically sampled flying foxes in each species had substantially lower odds of a positive HeV serostatus relative to immature opportunistically sampled flying foxes. Intuitively, this seems biologically nonsensical, as it suggests that the combination of being sick or injured (the source of the opportunistic sample) and mature age protects against HeV infection. However, the finding is consistent with infection in mature flying foxes (but not immature flying foxes) being associated with clinical disease and debility, and thus an increased probability of coming into care *prior* to seroconversion. Experimental studies indicate that seroconversion in flying foxes occurs between 14 and 21 days post-inoculation (Williamson *et al*, 1998; Williamson *et al*, 2000),

so it is plausible that naturally infected flying foxes coming into care in the 2-3 weeks post-infection could be seronegative. The lower likelihood of clinial disease in immature opportunistically sampled flying foxes could reflect a lower infection and disease prevalence associated with passive immunity. This hypothesis warrants testing, because if true, it offers an important addition to our understanding of the pathogenesis of HeV infection in flying foxes, as clinical disease is not currently recognised as a feature of infection in flying foxes.

Interpretation of the association between *SampleLocation* and *HeV serostatus* is less challenging. While it seems counterintuitive that seroprevalence would vary between locations in nomadic species, the multivariate analysis showed that the odds of antibodies to Hendra virus were 4.35 times as high in flying foxes surveyed in Queensland and New South Wales than in Western Australia and the Northern Territory. The findings are consistent with a pattern of pulsing endemicity (Thrusfield, 1986) in which infection and outbreaks occur in different parts of the range at different times. The association between *SampleYear* and *HeV serostatus* further supports this interpretation, and is consistent with infection occurring in sub-populations of flying foxes in a spatial and temporal mosaic. However, as non-random sampling techniques were used in the survey, sampling bias cannot be excluded from the deliberations, although the strength of the association, and the collection of samples at multiple locations and times in both regions mitigate against this. Another possible explanation is that the association is confounded by an important but unmeasured variable of host, agent or environment. Factors such as colony size and density, virus virulence, and seasonal variation in flying fox breeding cycles could all plausibly influence the rate of transmission between flying foxes.

The univariate association between time of year and HeV serostatus is also interesting. The analysis suggests a seasonal pattern of infection that varies with species. *P. alecto* are more likely to be seropositive in the first, third and fourth quarters, while *P. poliocephalus* and *P. scapulatus* are more likely to be seropositive in the second quarter. Translated into biological events, the findings suggest that *P. alecto* has an increased likelihood of HeV infection associated with late pregnancy (third quarter)[12], birthing (fourth quarter), and the post-natal period (first quarter); that *P. poliocephalus* has an increased likelihood of infection associated with mating and early pregnancy (second

[12] This association is not quite significant at the nominal 0.05 level, with an OR=1.96 (95%CI 0.94, 4.1) and p=0.07.

quarter); and that *P. scapulatus* has an increased likelihood of infection associated with its birthing season. The different temporal pattern of infection between species is also consistent with the population heterogeneity model of measles persistence favoured by Bolker and Grenfell (1995) and others. They contend that infection can persist in a population of heterogenous age, social or spatial structure by affecting different population cohorts over time. This hypothesis is discussed further in relation to the maintenance of Hendra virus infection in flying fox populations in Chapters 6 and 7. Notwithstanding the above discussion, the possibility that the association between time of year and HeV serostatus is due to chance (this is a non-random sample) or confounded by another variable cannot be ignored. While confounding by location and species are controlled (the former by restricting the analysis to southeast Queensland data; the latter by stratification), possible confounding by the other explanatory variables is not. That said, the magnitude of the association (reflected by the odds ratio) suggests the association is real, although the very high OR for *P. scapulatus* in the second quarter (27.60) and the wide 95% confidence interval (1.64 to 1477.84) reflect the limited numbers in this quarter.

3.4 INVESTIGATIONS OF OTHER WILDLIFE SPECIES FOR EVIDENCE OF HENDRA VIRUS INFECTION

3.4.1 Introduction

While seroepidemiology, virus isolation and experimental infection findings support the hypothesis that flying foxes are a natural host of Hendra virus, the novel nature of the virus warranted a more comprehensive investigation of possible wildlife reservoirs. The possibility of another host, or an intermediate host could not be discounted, particularly given the lack of an intuitive epidemiological link between flying foxes and horses. This line of reasoning was supported by a positive PCR finding in a pooled sample of mosquitoes from the Hendra index case paddock in 1995 (AR Gould, AAHL: pers. comm).

Five groups were targeted for further surveillance: other bat species, small terrestrial mammals, snakes and birds that shared habitat with flying foxes and horses, and mosquitoes. If flying foxes were a natural host, it was plausible that other bat groups could be also. As previously discussed, a report by Campbell *et al* (1977) of a novel

paramyxovirus in native rodents in 1970 influenced the focus of the initial wildlife surveillance. It now prompted the inclusion of rodents and other small terrestrial mammals in this survey, given that rodents in particular are frequently found around horses. Pythons (Family *Boidae*), for which flying foxes and rodents are common prey species, were also targeted. Ibis (Family *Plataleidae*) and egrets (Family *Ardeidae*) commonly share roosts with flying foxes. Ibis in particular were observed or reported to be frequently present in the immediate vicinity of horses in the Hendra and Mackay index paddocks. While no precedent exists for the transmission of paramyxoviruses by mosquitoes or other biting insect, the novel nature of the virus, the frequent location of flying fox camps in mosquito-rich mangrove habitats, and the plausible mode of mechanical transmission which mosquitoes represent argued for their inclusion.

The objective of this study, to screen targeted wildlife species for evidence of Hendra virus infection, was pursued through five parallel studies whose surveillance focus was the above groups.

3.4.2 Materials and methods

3.4.2.1 Study design

The surveys were conducted using a cross-sectional study design at the individual animal level. The study population comprised non-randomly (convenience or purposive) selected populations in Queensland (Brisbane, Cairns, Gold Coast, Karumba, Mt Glorious, Rockhampton, Yeepoon), New South Wales (Armidale), and Western Australia (Mitchell Plateau).

3.4.2.2 Sampling locations

Other bat species

Populations of ubiquitous insectivorous bat species in or near urban areas were sampled at multiple locations in eastern Queensland: multiple sites in suburban Brisbane in house ceilings, stormwater drains or peri-urban abandoned mines (Common Bent-wing bat *Miniopterus schreibersii*, Little Broad-nosed bat *Scotorepens greyii*, Little Northern Mastiff-bat *Mormopterus loriae*, Beccari's Mastiff-bat *Mormopterus becarrii*), a riparian sandstone cave in forest near Yeepoon (Little Bent-wing bat *Miniopterus australis*), and in suburban Cairns in house ceilings (Little Northern Mastiff-bat *Mormopterus loriae*).

Terrestrial mammal species

The sample is predominantly from an archived collection previously collected for an unrelated study[13]. They were originally obtained from free-living populations in primarily forested areas near Armidale (Common Ringtail Possum *Pseudocheirus peregrinus*), Mt Glorious (Brown Antechinus *Antechinus stuartii*, Bush Rat *Rattus fuscipes*, Fawn-footed Melomys *Melomys cervinipes*), and Mitchell Plateau (Common Rock-rat *Zyzomys argurus*, Grassland Melomys *Melomys burtoni*, Northern Brown Bandicoot *Isoodon macrourus*, Northern Quoll *Dasyurus hallucatus*, Pale Field-rat *Rattus tunneyi*, Scaly-tailed possum *Wyulda squamicaudata*, unspeciated rats *Rattus spp.*). In addition, a contemporary sample was collected from free-living populations in suburban Brisbane (Black Rat *Rattus rattus*, Brown Rat *Rattus norvegicus*, House Mouse *Mus musculus*) and Cairns (Black Rat *Rattus rattus*), and from a captive population in Brisbane (Tammar Wallaby *Macropus eugenii*)

Avian species

Populations of waterfowl and wader species were sampled at multiple locations in Queensland: an intertidal site in Brisbane (Red-necked Stint *Calidris ruficollis*, Curlew Sandpiper *Calidris ferruginea*, Bar-tailed Godwit *Limosa lapponica*, Gull-billed Tern *Gelochelidon nilotica*), an urban refuse tip on the Gold Coast (White Ibis *Threskiornis molucca*), wetland and intertidal sites near Karumba (Pied Heron *Ardea picata*, Great Knot *Calidris tenuirostris*, Red Knot *Calidris canutus*, Greater Sand-plover *Charadrius leschenaultii*), and suburban Rockhampton (Grass Whistling Duck *Dendrocygna eytoni*, Black duck *Anas superciliosa*).

Snake species

Boid (Family *Boidae*) and colubrid (Family *Colubridae*) snakes were non-randomly sampled at multiple (primarily forested) locations in northern Queensland: Paluma (*Boiga irregularis*, *Liasis maculosus*, *Morelia amethistina*, *Stegonotus cucullatus*, *Tropidonophis mairii*), Babinda (*Boiga irregularis*, *Morelia amethistina*, *Morelia spilota*), Tully Gorge (*Boiga irregularis*, *Morelia amethistina*, *Stegonotus cucullatus*), Bramston Beach (*Boiga irregularis*, *Morelia amethistina*, *Stegonotus cucullatus*), and Goldsborough (*Boiga irregularis*, *Liasis maculosus*). In addition, captive populations on the Gold Coast were sampled (*Morelia spilota*, *Morelia sp.*, *Liasis fuscus*, *Liasis olivaceus*).

[13] Conducted by Dr Adrian Bradley, Dept Physiology and Pharmacology, The University of Queensland.

Insects
Mosquitoes were trapped at three locations; in suburban Brisbane (Indooroopilly) in riparian mangrove habitat; in suburban Cairns in melaleuca wetland; and in the lightly timbered Trinity Beach paddock of the Cairns case horse. Haematophagus obligate ectoparasites of flying foxes (*Cyclopodia albertisii*. family *Nycteribiidae*), commonly known as nycteribids, were collected from *P. alecto* in Townsville in north Queensland, from *P. alecto* and *P. poliocephalus* at Indooroopilly Island and other locations in the greater Brisbane area, and from *P. scapulatus* in Munduberra in central Queensland.

3.4.2.3 Sampling methodology

Other bat species
Bats were non-randomly (convenience) sampled by trapping in mist-nets or harp traps set in observed or likely flight paths, or in caves or old mines, by hand net. Mist-nets were set at dusk and monitored continuously for 2-3 hours after dusk. Harp traps were set at dusk and inspected early the following day. Captured bats were euthanased by CO^2 asphyxiation for parallel Australian bat lyssavirus studies.

Terrestrial mammal species
There were two sampling methodologies. An archived serum collection was accessed, the original collection methodology of which is unknown. All available archived samples were screened. Secondly, rodents were non-randomly (convenience) sampled by trapping using Elliott™ traps baited with either a stiff mixture of peanut paste and rolled oats, or a commercial dry dog food. Trap lines, positioned in locations of known or likely habitat, were laid out at dusk and collected early the following morning. Rodents were euthanased by CO2 asphyxiation for parallel hantavirus studies.

Avian species
Bird populations were non-randomly (convenience) sampled by cannon-netting in conjunction with the Southeast Queensland Wader Study Group banding activities, or with strategic AQIS surveillance activities. Nets were set in observed foraging or roosting sites at the various locations. Netted birds were placed in portable hessian or shadecloth cages and processed expeditiously. The number of birds caught at one time was limited to ensure holding times were minimised. All birds were released.

Snake species

Snakes were non-randomly (convenience) sampled by experienced herpetologists for an unrelated study[14]. The capture methodology used is unknown. Blood samples were collected and all snakes released.

Insects

Mosquitoes were non-randomly (convenience) sampled at various locations in the vicinity of known flying fox roosting or feeding sites. CO_2/light traps were set at dusk and recovered early the following morning. Mosquitoes were killed by refrigeration prior to sorting (on blood-fed status) and speciation. Nycteribids were non-randomly (convenience) sampled from free-living flying foxes and killed by refrigeration.

3.4.2.4 Sample size

Minimum sample size targets were consistent for all surveys. A minimum sample size of 20 individuals per species was sought, enabling detection of antibodies (with 95% statistical confidence) at a minimum population seroprevalence of 15%, assuming 100% test sensitivity and specificity.

3.4.2.5 Sampling period

Other bat species

Bats were surveyed between October 1996 and August 1999.

Terrestrial mammal species

Archived specimens were collected between 1982 and 1984. The contemporary rodent specimens were collected between October 1998 and February 1999. The captive Tammar wallaby colony was bleed in December 1997.

Avian species

Avian samples were collected between September 1996 and April 1998.

Snake species

Snakes were captured and bleed between September and November 1998.

[14] Conducted by a post-graduate student of Dr. Joan Whittier, School of Veterinary Science, The University of Queensland.

Insects

Mosquitoes and nycteribids were sampled intermittently between May 1995 and June 1999.

3.4.2.6 Specimen collection

Other bat species

Blood was collected at euthanasia by cardiac puncture. The serum yield was stored at -20°C. Tissue samples (brain, lung, liver, kidney, spleen, testes or uterus) were also taken for Hendra virus isolation studies and for a concurrent survey of ABLV.

Terrestrial mammal species

Frozen sera were available from the archived collection. Trapped rodents were euthanased and blood collected by cardiac puncture. Serum was harvested and stored at -20°C.

Avian species

Limited-volume blood samples (not exceeding 5% of estimated blood volume) were collected from the jugular or radial veins by venipuncture using a heparinized syringe. Plasma was harvested and stored as above.

Snake species

Similarly limited-volume blood samples were collected from the coccygeal vein by venipuncture using a heparinized syringe. Plasma was harvested and stored as above.

Insects

Blood-fed mosquitoes and nycteribids were collected by CO_2/light trap and by hand respectively, and stored at -20°C.

3.4.2.7 Laboratory methodologies

All sera were forwarded to AAHL for screening by SNT. In addition to the SNT being the gold standard serologic test for Hendra virus, an absence of suitable conjugates for avian and reptile sera precludes ELISA methodology for these groups. Insects were homogenized and subject to PCR analysis for Hendra virus antigen at the YVL.

3.4.2.8 Data management and statistical analyses

All data were stored and managed in a Microsoft ™ Access 97 ™ database. Data was exported to Microsoft ™ Excel 97 ™ for descriptive analysis.

3.4.3 Results

Other bat species

A total of 225 bats from seven genera of insectivorous bats were screened. All sera were negative for antibodies to Hendra virus (Table 3.19).

Terrestrial mammal species

A total of 537 animals from 15 non-bat species were screened. All sera were negative for antibodies to Hendra virus (Table 3.20).

Table 3.19: Serosurveillance of 225 insectivorous bats sampled at multiple locations in Queensland between 1996 and 1999 for evidence of infection with Hendra virus.

Genus/species	Number of animals	
	Total	Testing positive[1]
Miniopterus spp.	62	0
Mormopterus spp.	53	0
Saccolaimus flaviventris	49	0
Taphozous spp.	46	0
Chalinobus spp.	7	0
Chaerophon jobensis)	5	0
Scotorepens greyii	3	0
Total	225	0

[1] by serum neutralisation test

Table 3.20: Serosurveillance of 15 mammalian wildlife species[1] sampled at multiple locations in northern Australia between 1984 and 1998 for evidence of infection with Hendra virus.

Genus/species	Number of animals	
	Submitted	Testing positive[2]
Northern Quoll (*Dasyurus hallucatus*)	113	0
Northern Brown Bandicoot (*Isoodon macrourus*)	94	0
Brown Rat (*Rattus norvegicus*)	62	0
Common Ringtail Possum (*Pseudocheirus peregrinus*)	61	0
Grassland Melomys (*Melomys burtoni*)	60	0
Scaly-tailed possum (*Wyulda squamicaudata*)	30	0
Brown Antechinus (*Antechinus stuartii*)	25	0
Fawn-footed Melomys (*Melomys cervinipes*)	20	0
Common Rock-rat (*Zyzomys argurus*)	17	0
Bush Rat (*Rattus fuscipes*)	12	0
House Mouse (*Mus musculus*)	13	0
Tammar Wallaby (*Macropus eugenii*)	10	0
Black Rat (*Rattus rattus*)	10	0
Unspeciated rats (*Rattus spp.*)	6	0
Pale Field-rat (*Rattus tunneyi*)	5	0
Total	538	0

[1] non-bat species
[2] by serum neutralisation test

Avian species

A total of 152 birds from 11 species were screened. All sera were negative for antibodies to Hendra virus (Table 3.21).

Snake species

A total of 73 snakes from 9 species were screened. All sera were negative for antibodies to Hendra virus (Table 3.22).

Insects

A total of 12 pooled samples of mosquitoes and 50 pooled samples of nycteribids were screened. All were negative for Hendra virus antigen (Table 3.23).

Table 3.21: Serosurveillance of 11 avian wildlife species sampled at multiple locations in Queensland between 1996 and 1998 for evidence of infection with Hendra virus.

Genus/species	Number of animals	
	Total	Testing positive[1]
White Ibis (*Threskiornis molucca*)	54	0
Pied Heron (*Ardea picata*)	27	0
Grass Whistling Duck (*Dendrocygna eytoni*)	18	0
Red-necked Stint (*Calidris ruficollis*)	11	0
Curlew Sandpiper (*Calidris ferruginea*)	11	0
Bar-tailed Godwit (*Limosa lapponica*)	10	0
Great Knot (*Calidris tenuirostris*)	6	0
Black duck (*Anas superciliosa*)	6	0
Red Knot (*Calidris canutus*)	3	0
Greater Sand-plover (*Charadrius leschenaultii*)	3	0
Gull-billed Tern (*Gelochelidon nilotica*)	3	0
Total	152	0

[1] by serum neutralisation test

Table 3.22: Serosurveillance of reptile wildlife species for evidence of infection with Hendra virus (1996-1998).

Genus/species	Number of animals	
	Total	Testing positive[1]
Eastern Brown Tree Snake (*Boiga irregularis*)	23	0
Carpet Python (*Morelia spilota*)	20	0
Amethyst Python (*Morelia amethistina*)	11	0
Slate-grey Snake (*Stegonotus cucullatus*)	8	0
Water Python (*Liasis fuscus*)	4	0
Eastern Small-blotched Python (*Liasis maculosus*)	2	0
Keelback Snake (*Tropidonophis mairii*)	2	0
Python hybrid (*Morelia sp.*)	2	0
Olive Python (*Liasis olivaceus*)	1	0
Total	73	0

[1] by serum neutralisation test

Table 3.23: Speciation of a sample of 596 mosquitoes caught in one trap at the Trinity Beach on 20/2/99.

Genus/species	Number
Culex annulirostris	42
Culex vicinus	5
Aedes lineatus	242
Aedes kochii	97
Aedes funereus	35
Aedes lineatopennis	8
Aedes notoscriptus	84
Aedes normanensis	1
Aedes vigilax	7
Aedes impremans	11
Aedes palmorum	17
Coquillettida xanthergaster	1
Mansonia septempunctata	42
Anopheles farauti	4
Total	596

3.4.4 Discussion

The earlier investigations of the origins of Hendra virus indicated that flying foxes were a natural host of Hendra virus. However a mode of transmission from flying foxes to horses was not identified, leaving open the possibility that another natural host or an intermediate host species was the source of infection in horses. This study found no serologic evidence of Hendra virus in a total of 988 animals from 22 families. Mindful of the interpretative limitations of negative findings inherent in ad hoc wildlife surveys (discussed in Section 3.2.4), the current study sought to maximise the sensitivity of the survey by positively biasing the sample to include species that shared habitat with flying foxes and horses. In addition, the minimum sample size per species was determined assuming a conservative 15% seroprevalence. Based on that apparent in flying foxes, it was assumed that any other significant host would have a similar seroprevalence. While it could be argued that the inclusion of archived sera from geographic locations remote from known outbreaks of HeV in horses lessens the sensitivity of the survey, the temporal and spatial origins of these samples were encompassed by widespread occurrence of HeV in Australian flying foxes described in Section 2.5.3. Of the 43 plus vertebrate species surveyed, the minimum sample size was attained in 17. The absence of anti-HeV

antibodies in surveyed individual of these species is interpreted as evidence of absence of infection in the sampled populations.

While true insect-vectored transmission is unknown in viruses of the family *Paramyxoviridae*, it is plausible that haematophagus insects could transmit Hendra virus infection mechanically. Flying fox camps are commonly located in littoral or tidal locations where mosquitoes breed, and flying foxes have obligate blood-feeding ectoparasites (nycteribids) that can readily move between roosting individuals. Thus, mosquitoes and nycteribids have the opportunity to mechanically transmit infection between flying foxes, and mosquitoes could plausibly transmit infection from flying foxes to horses. An earlier positive Hendra virus PCR product in a pooled sample of blood-fed mosquitoes collected from the Brisbane index case paddock at Cannon Hill in late 1994 or early 1995 (AR Gould, AAHL; pers. comm.) argued for further investigation of insects. The screening of mosquitoes and nycteribids in this study failed to detect any positive samples. However it is premature to conclude that these or other haematophagus insects play no role in transmission. The study was limited both in sample size and sample location, and the presence or absence of infection in the local flying fox population at the time of sampling was unknown.

If it is concluded that flying foxes are the origin of infection in horses, the question of the mode of transmission remains. Several hypotheses have been proposed; the ingestion by horses of pasture contaminated with the urine of an infected flying fox; the ingestion of pasture contaminated with foetal fluids or tissues; and the ingestion of the masticated pellets of residual fruit pulp spat out by flying foxes. Despite the substantial distributional overlap of horse and flying fox populations in Australia, the spillover of Hendra virus to horses is a rare event. This argues for an obscure mode of transmission, or alternatively a complex causal model. The mode of transmission of HeV infection to horses will be discussed further in Chapter 7.

3.4 CHAPTER CONCLUSIONS

The preliminary wildlife investigations demonstrated the challenges associated with surveillance of uncontrolled wildlife populations. The sample is rarely random. Its representativeness is generally unknown. The capture methodology used largely dictates

the potential sample composition. A 'catch-all' approach means that trap catch will reflect relative abundance, and that rare species will be under-represented. The decision in this study to sample sick and injured free-living wildlife in temporary captivity led to the identification of serum neutralising antibodies to Hendra virus in flying-foxes. This finding was a major breakthrough in the search for the origin of Hendra virus and precipitated investigation of the role of flying foxes in the ecology of the virus. These subsequent studies showed evidence of previous exposure to Hendra virus in flying foxes across their Australian range, with species, location, year, and age being risk factors for infection. Further, evidence of infection was apparent in archived sera from two species of flying fox in southeast Queensland, establishing that infection in flying foxes did not immediately precede infections in horses and humans. These features suggested a major role for flying foxes in the ecology of Hendra virus, and are consistent a mature host-agent relationship. Notwithstanding, a number of questions remain to be answered. What is the primary route of excretion and the mode of transmission in flying foxes? What is the mode of transmission to horses? Could an intermediate host be involved? The final study in this chapter found no evidence of Hendra virus infection in a wide range of taxa, supporting the contention that flying foxes are not only a natural host of Hendra virus, but likely the reservoir of infection and the source of infection for horses.

As previously discussed, Mills *et al* (1998) contend that a comprehensive investigation of a reservoir host should include 1) an understanding of the ecology of the natural host, 2) definition of the distribution of the agent within the host range, 3) identification of any ecological variables influencing agent and host distribution, 4) identification of significant host-level variables, 5) longitudinal studies to enable a temporal perspective, and 6) the development of predictive models. Fortunately, the first point is largely addressed by the substantial existing body of knowledge of the ecology of Australian flying foxes (Eby, 1991; Hall & Richards, 2000; Marshall, 1985; Richards, 1990a; Richards, 1990b; Webb & Tidemann, 1996). The investigations presented in this chapter have largely focused points two, three and four; defining the spatial occurrence of Hendra virus infection in flying foxes, and identifying animal-level and external risk factors for infection. However, questions on the dynamics of infection and the mode of transmission in flying foxes remain unanswered. Longitudinal studies and predictive modelling (points five and six above) presented in Chapters 4 and 6 respectively seek to address these and other questions.

CHAPTER 4

INVESTIGATIONS OF THE TRANSMISSION AND MAINTENANCE OF HENDRA VIRUS IN FLYING FOXES – a longitudinal study series.

4.1 Introduction

The cross-sectional study series presented in Chapter 3 initially identified evidence of Hendra virus infection in flying foxes, and subsequently the spatial occurrence of infection and risk factors for infection in flying foxes. In conjunction with the experimental studies conducted at AAHL (Chapter 2), the studies provided strong evidence that flying foxes were a natural host and a plausible reservoir of HeV. However, the means by which infection was transmitted and maintained in flying fox populations remained unknown. The studies described in this chapter sought to address this deficiency.

Specifically, the studies aimed to detect new infections, to identify routes of virus excretion, and to identify maternal antibody transmission and persistence in a captive flying fox population.

4.2 Materials and methods

4.2.1 Study design

The study population comprised a captive colony of flying foxes. Individual animals were the unit of interest. Initially, the study population was cross-sectionally surveyed for neutralising antibodies to Hendra virus (Study 1); secondly, a sample of the population was prospectively monitored for virus and for seroconversion (Study 2); thirdly, a sample of the population was prospectively monitored serologically (Study 3). Studies 2 and 3 had staggered entry and exit dates.

4.2.2 Study location

A captive colony of flying foxes existed at a UQ research facility at Pinjarra Hills, Brisbane. The colony had been established from wild-caught flying foxes in the mid-1980s

for reproduction physiology research[15], and included *P. alecto* and *P. poliocephalus*. This original population was progressively augmented by natural increase, by the periodic introduction of rehabilitated sick and injured wild flying foxes, and the introduction of surplus flying foxes from other captive colonies. At the commencement of Study 1, the colony comprised 100 flying foxes from two species, with an estimated age range of five months to ten years. A group of five *P. conspicillatus* was introduced from another captive colony immediately prior to the commencement of Study 3.

4.2.3 Sample selection and size

Study 1

A non-random convenience sample of 68 flying foxes was selected from the study population.

Study 2

A non-random purposive sample of 17 *P. poliocephalus* was selected from those screened in Study 1. All were uniquely identified by microchip or thumb-band number.

Study 3

A non-random convenience sample of 54 flying foxes was non-randomly selected from the study population. Five were previously enrolled in Study 2. All were uniquely identified by microchip.

4.2.4 Study period

Study 1 24th May to 11th June 1996.

Study 2 29th August 1996 to 27th November 1996

Study 3 31st January 1997 to 15th December 1998.

4.2.5 Specimen collection

Individuals were caught, wrapped firmly in a thick towel, and physically restrained in dorsal recumbency using a purpose-built device[16]. This technique left only the head and

[15] The colony was established by Dr Len Martin, then at the UQ Department of Physiology and Pharmacology.

[16] People handling flying foxes observed DPI&F Workplace Health & Safety protocols: they were experienced handlers, wore the proscribed personal protective equipment, were rabies-vaccinated, and were subject to six-monthly monitoring of post-vaccination antibody titre. See Appendix 1.

the hind limbs exposed, facilitating the ready collection of a blood and/or swab samples. All samples were forwarded to YVL.

Blood (Studies 1, 2 & 3)

In Study I, enrolled individuals were bled on a single occasion; in Study 2, blood was collected weekly from each individual; in Study 3, blood was collected monthly from each individual. Typically, one millilitre (ml) of blood was taken from a vein in the uropatagium on the medial aspect of the hind limb using a 23-gauge needle and a heparinised one or two-millilitre syringe. In neonates under two months of age, 0.3 – 0.5 ml of blood was collected by stabbing the cephalic vein (running along the leading edge of the wing) with a 21-gauge needle and collecting drops of blood into a paediatric EDTA tube.

Swabs (Study 2)

Individuals were sampled weekly. Paediatric swabs were used to collect throat and urogenital swabs. These were placed into one millilitre of a standard virus transport medium (phosphate-buffered saline with added antibiotic and antifungal agents).

4.2.6 Laboratory methods

Serology (Studies 1, 2 & 3)

Plasma was harvested from the blood samples by centrifugation. An aliquot was forwarded to AAHL for testing for antibodies to Hendra virus by SNT. Individuals with neutralising antibody titres of 1:5 and above were classed as positive; those with titres <1:5 were classed as negative.

Virus isolation (Study 2)

Washed red blood cells and the throat and urogenital swabs were subjected to cell culture for virus isolation using RK13 cells[17]. Cultures were observed daily for CPE. If none was observed over seven days, the inoculated cultures were harvested by a freeze-thaw and passaged. This process was repeated for a maximum of five serial passages (Halpin *et al.*, 2000).

[17] Cell culture was undertaken by Dr Kim Halpin (2000) as part of her investigation of the comparative virology of Hendra virus.

4.2.7 Data management and analysis

Data were recorded on seven animal-level variables (animal ID, species, sex, date of enrolment, age at enrolment, and date of exit) and three sample variables (date collected, HeV neutralising antibody titre and HeV virus isolation result). Three age classes were defined: immature (less that 30 months), mature, and aged (greater than 10 years). As previously described, classification was based on morphometric and physiological criteria including weight and forearm length, dentition wear, and mammary development. Maturity (defined as sexual maturity) is attained by the majority of flying foxes in the second breeding season after their birth (that is, at 30 months (Hall & Richards, 2000; McIllwee & Martin, 2002). Flying foxes were classified as aged if molar teeth were bilaterally worn to the gum or lost. These individuals were estimated to be greater than 10 years old (LS Hall, School of Veterinary Science, UQ; pers. comm.). All data were stored and managed in a relational database using Microsoft ™ Access 97 ™. Data were exported to Microsoft ™ Excel 97 ™ for descriptive and univariate analyses. EpiInfo (Version 6) Statcalc was used in the latter to test for associations between each of the outcome variables and the possible explanatory variables, with Chi square *p* values indicating the statistical significance of associations, and unadjusted relative risk (RR) indicating the magnitude of associations[18]. Yates Corrected *p* value was used except where cell values were less than 5, when Fisher's Exact *p* value was used.

4.3 Results

Study I

Neutralising antibody titres to Hendra virus were found in 19% of the 68 flying foxes surveyed: 9/55 *P. poliocephalus* and 4/13 *P. alecto* (Table 4.1). Titres ranged from 1:5 to 1:160 (See Appendix 2), with a median of 1:10.

Study 2

Blood, and throat and urogenital swabs were collected weekly for 14 weeks from between 10 and 17 *P. poliocephalus*. Seronegative, mature-age females (15, 13 and 12 respectively) were purposely over-represented (See Appendix 3). A total of 556 specimens (190 blood samples, 183 throat swabs and 183 urogenital swabs) were collected (Table 4.2). No flying foxes seroconverted during the study period. Virus was isolated on one

[18] Relative risk was used in preference to odds ratio because the former is more readily and widely interpretable, and because stratified analysis (which does not require the use of odds ratios) was used for multivariable analysis.

occasion, an observed incidence rate of one case per 3.65 animal-years at risk[19]. The isolate was recovered from the blood of an aged non-pregnant female (BR153). This individual was negative for virus and antibody at previous bleeds and at the two subsequent bleeds, after which she was euthanased. At necropsy, virus was not evident in liver, lung, kidney, spleen and uterus by cell culture or by RT-PCR. The presence of virus in the original sample was subsequently reconfirmed by cell culture and by RT-PCR (Kim Halpin; pers. comm.)

Table 4.1: Characteristics of 68 flying foxes in a captive colony screened for neutralising antibodies to Hendra virus in June 1996.

Variable	Number of flying foxes	
	Tested	With neutralising antibodies to HeV
Species		
P. poliocephalus	55	9
P. alecto	13	4
Sex		
Male	24	6
Female	44	7
Age		
Immature	16	5
Mature	12	1
Unknown	40	7
Total	68	13

[19] 190 animal-weeks is equivalent to 3.65 animal-years.

Table 4.2: Virus isolation outcomes in 17 flying foxes in a captive colony screened for Hendra virus between August and November 1996.

Week #	Bleed date	Number of individuals tested (positive) for virus in		
		Blood	Throat swab	Urogenital swab
1	29/08/96	12	12	12
2	05/09/96	12	12	12
3	11/09/96	10	10	10
4	18/09/96	10	10	10
5	25/09/96	12	12	12
6	02/10/96	10	10	10
7	09/10/96	10	10	10
8	16/10/96	12 (1)	12	12
9	23/10/96	17	17	17
10	30/10/96	17	16	16
11	06/11/96	17	16	16
12	13/11/96	17	12	12
13	20/11/96	17	17	17
14	27/11/96	17	17	17
Total		190 (1)	183	183

Study 3

A total of 54 flying foxes was enrolled for all or part of the study period. Of these, 19 were HeV seropositive at entry (Table 4.3) and 35 were seronegative at entry (Table 4.4). Univariate analysis indicated a strong association between species and HeV serostatus at entry, with spectacled flying foxes a little over 4 times more likely (95% CI 2.5 to 7.0) to be seropositve at entry than grey-headed flying foxes (Table 4.5). Three individuals that were seronegative on entry became seropositive during the study (Table 4.6). All three were pups born during the study period. Three flying foxes that were seropositive on entry became seronegative subsequently (Table 4.7). Two of these were pups born during the study period.

Table 4.3: Characteristics of 19 flying foxes in a captive colony screened for neutralising antibodies to Hendra virus between January 1997 and December 1998 that were seropositive at entry.

ID	Species	Sex	Date of entry	Age class at entry	Reciprocal HeV titre at entry	Date of Exit
00013204A0	*P. conspicillatus*	Female	31.1.97	Mature	160	1.4.98
0001DB945F	*P. conspicillatus*	Female	31.1.97	Mature	20	13.8.98
0001DC4B7A	*P. conspicillatus*	Female	31.1.97	Immature	40	21.10.98
0001DC4051	*P. conspicillatus*	Male	31.1.97	Mature	320	1.4.98
0001DB0B25	*P. conspicillatus*	Male	26.2.97	Mature	80	3.12.97
0001DB044F[a]	*P. conspicillatus*	Male	31.1.97	Mature	5	3.12.97
41054D7E52	*P. conspicillatus*	Male	7.11.97	Immature	320	15.12.98
41054C4E3B	*P. conspicillatus*	Male	25.11.97	Immature	>640	15.12.98
0001232432	*P. poliocephalus*	Female	7.2.97	Aged	40	13.8.98
0001DAD405	*P. poliocephalus*	Female	26.2.97	Mature	40	1.4.98
000134EEA6	*P. poliocephalus*	Female	7.2.97	Mature	10	13.8.98
000134F4DB	*P. poliocephalus*	Female	7.2.97	Mature	10	13.8.98
00013C709A	*P. poliocephalus*	Female	7.2.97	Mature	80	1.4.98
0001D38B13	*P. poliocephalus*	Female	7.2.97	Mature	5	13.8.98
0001435949	*P. poliocephalus*	Female	8.4.97	Mature	5	22.12.97
0001DAE2AC	*P. poliocephalus*	Female	7.2.97	Immature	20	8.4.97
410666530E[a]	*P. poliocephalus*	Female	25.11.97	Immature	160	22.12.97
0001DB05DC	*P. poliocephalus*	Male	26.12.97	Mature	10	1.4.98
0001FB41CA[a]	*P. poliocephalus*	Male	7.2.97	Immature	40	21.10.98

[a] Individuals seropositive on entry that subsequently became seronegative. See Table 4.6 for serial titres.

Table 4.4: Characteristics of 35 flying foxes in a captive colony screened for neutralising antibodies to Hendra virus between January 1997 and December 1998 that were seronegative at entry.

ID	Species	Sex	Date of entry	Age class at entry	Date of Exit
0001DC2FA0	*P. poliocephalus*	Female	7.2.97	Aged	22.12.97
0001DC4147	*P. poliocephalus*	Female	12.3.97	Aged	22.12.97
0001D38C92	*P. poliocephalus*	Female	7.2.97	Mature	22.12.97
0001DB8BE0	*P. poliocephalus*	Female	13.2.97	Mature	3.12.97
0001DB8B51	*P. poliocephalus*	Female	26.3.97	Mature	7.11.97
0001D302AE	*P. poliocephalus*	Female	13.2.97	Mature	22.12.97
0001D3B20E	*P. poliocephalus*	Female	31.1.97	Mature	3.12.97
0001339DA2	*P. poliocephalus*	Female	31.1.97	Mature	3.12.97
0001351863	*P. poliocephalus*	Female	31.1.97	Mature	3.12.97
0001435C30	*P. poliocephalus*	Female	31.1.97	Mature	3.12.97
0001447F33	*P. poliocephalus*	Female	7.2.97	Mature	22.12.97
0001D1EBAD	*P. poliocephalus*	Female	7.2.97	Mature	22.12.97
0001BB7D58	*P. poliocephalus*	Female	7.2.97	Mature	22.12.97
0001FB8E71	*P. poliocephalus*	Female	7.2.97	Mature	22.12.97
0001D2833A	*P. poliocephalus*	Female	31.1.97	Mature	3.12.97
0001216DF7 [b]	*P. poliocephalus*	Female	7.2.97	Immature	3.12.97
0001D23559	*P. poliocephalus*	Female	7.2.97	Immature	22.12.97
000121D019	*P. poliocephalus*	Female	7.2.97	Immature	3.12.97
0001DB824E	*P. poliocephalus*	Female	31.1.97	Immature	3.12.97
41065C567C [b]	*P. poliocephalus*	Female	25.11.97	Immature	13.8.98
0001FB47B9	*P. poliocephalus*	Male	7.2.97	Aged	22.12.97
0001235B79	*P. poliocephalus*	Male	7.2.97	Aged	22.12.97
00012274BE	*P. poliocephalus*	Male	7.2.97	Mature	22.12.97
00013D6207	*P. poliocephalus*	Male	31.1.97	Mature	3.12.97
000134EBC0	*P. poliocephalus*	Male	31.1.97	Immature	3.12.97
0001D34CE1	*P. poliocephalus*	Male	31.1.97	Immature	3.12.97
0001133AAF	*P. poliocephalus*	Male	7.2.97	Immature	22.12.97
0001DB0BDE	*P. poliocephalus*	Male	26.2.97	Immature	3.12.97
000121CFC8	*P. poliocephalus*	Male	7.2.97	Immature	22.12.97
0001DB069C	*P. poliocephalus*	Male	31.1.97	Immature	3.12.97
0001353FBD	*P. poliocephalus*	Male	7.2.97	Immature	22.12.97
00013D3AFA	*P. poliocephalus*	Male	7.2.97	Immature	22.12.97
0001DB83A2	*P. poliocephalus*	Male	7.2.97	Immature	22.12.97
410656613C	*P. poliocephalus*	Male	25.11.97	Immature	22.12.97
1055A0437 [b]	*P. poliocephalus*	Male	25.11.97	Immature	13.8.98

[b] Individuals that subsequently seroconverted. See Table 4.7 for serial titres.

Table 4.5: Univariate association between a number of independent variables and HeV neutralising antibody status at entry in 54 non-randomly sampled flying foxes in a captive colony between January 1997 and December 1998.

Variable	Number of flying foxes		P value	Relative risk (95%CI)
	Seropositive at Entry	Seronegative at Entry		
Species			<0.001	
P. poliocephalus	11	35		Referent
P. conspicillatus	8	0		4.18 (2.5, 7.0)
Age			0.35	
Immature	6	16		Referent
Mature	12	15		1.63 (0.73, 3.63)
Aged	1	4		0.77 (0.11, 4.81)
Sex			0.89	
Male	7	15		Referent
Female	12	20		1.18 (0.55, 2.51)

Table 4.6: Serial reciprocal titres of three serologically monitored flying foxes that were seronegative for neutralising antibodies to HeV on entry and became seropositive during the study period.

Date		Flying fox microchip number		
		41055A0437	41065C567C	0001216DF7
1997	Jan			
	Feb			<5
	Mar			10
	Apr			<5
	May			5
	June			5
	July			<5
	Aug			<5
	Sept			5
	Oct			<5
	Nov	<5	<5	<5
	Dec	320	40	<5
1998	Jan			
	Feb	40	10	
	Mar	80	5	
	Apr	160	5	
	May	80	80	
	June	80	0	
	July	40	5	
	Aug	20	0	
	Sept	80	40	
	Oct	40	0	
	Nov	10	5	
	Dec	20	5	

Table 4.7: Serial reciprocal titres of three serologically monitored flying foxes that had neutralising antibodies to HeV on entry and became seronegative during the study period.

Date		Flying fox microchip number		
		410666530E	0001DB044F	0001FB41CA
1997	Jan		5	
	Feb		<5	40
	Mar		<5	
	Apr		5	5
	May		<5	20
	June		<5	<5
	July		<5	20
	Aug		<5	10
	Sept		<5	5
	Oct		<5	<5
	Nov	160	<5	<5
	Dec	<5	<5	<5
1998	Jan			
	Feb			<5
	Mar			<5
	Apr			<5
	May			<5
	June			<5
	July			<5
	Aug			<5
	Sept			<5
	Oct			<5
	Nov			
	Dec			

Seventeen pups borne during the study were monitored (Table 4.8). Their ages on entry ranged from 2-6 weeks. Eleven were born in the 1996-97 season and six were born in the 1997-98 season. Nine pups were born to five seropositive dams and eight pups were born to six seronegative dams. Of the nine pups born to seropositive dams, five were seropositive at their first bleed, and a further three (seronegative at their first bleed) were seropositive at their second bleed (at either 30 days (1) or 60 days (2) post-entry). The ninth pup was seronegative at all bleeds. Of the eight pups born to seronegative dams, one was seropositive at the first bleed, but was seronegative at the second bleed (30 days post-entry); the other seven pups were seronegative at every bleed. Dam serostatus and pup serostatus at second bleed were strongly associated when data from

both seasons were combined ($p<0.001$; RR=9, 95%CI 1.42 to 57.12). The sparseness of the data precluded meaningful analysis of the two seasons separately.

Nineteen of the 54 flying foxes in the study were monitored for 12 months or longer. The serial titres of this cohort were examined for a within-year pattern. Three patterns were observed: rising and falling (defined as a fourfold or greater increase from the initial titre followed by a fourfold or greater decrease), falling (defined as greater than a fourfold decrease from the initial titre), and static (defined as less than a fourfold variation in either direction from the initial titre). Ten of the nineteen flying foxes exhibited a rising and falling pattern (Table 4.9), one of nineteen a static pattern (Table 4.10), and eight of nineteen a falling pattern (Table 4.11). The latter group included all seven pups monitored over the period, three born in the 1996 season and four in the 1997 season.

Table 4.8: Characteristics of 17 flying fox pups with dams of known HeV serostatus screened for neutralising antibodies to HeV between February 1997 and December 1998.

Year of Birth	Pup ID	Reciprocal HeV titre at Entry	Reciprocal HeV titre at second bleed [c]	Dam ID	Dam serostatus	Species
1996	0001DC4B7A	40	40	0001DB945F	Positive	*P. consp*
	0001DAE2AC	20	10 [c]	000134F4DB	Positive	*P. polio*
	0001FB41CA [a]	40	5 [c]	000134EEA6	Positive	*P. polio*
	0001216DF7 [b]	<5	10	0001D38B13	Positive	*P. polio*
	0001353FBD	<5	<5	0001232432	Positive	*P. polio*
	0001DB069C	<5	<5	0001C9938A	Negative	*P. polio*
	0001DC4DD2	<5	<5	0001339DA2	Negative	*P. polio*
	0001D23559	<5	<5	0001D38C92	Negative	*P. polio*
	0001133AAF	<5	<5	0001DC2FA0	Negative	*P. polio*
	000121CFC8	<5	<5	0001BB7D58	Negative	*P. polio*
	00013D3AFA	<5	<5	0001D1EBAD	Negative	*P. polio*
1997	41054D7E52	320	160	0001DB945F	Positive	*P. consp*
	41065C567C [b]	<5	40	000134F4DB	Positive	*P. consp*
	41055A0437 [b]	<5	320	0001232432	Positive	*P. consp*
	41054C4E3B	>640	80	0001D38B13	Positive	*P. consp*
	410666530E [a]	160	<5	0001D38C92	Negative	*P. polio*
	410656613C	<5	<5 [c]	0001DC2FA0	Negative	*P. polio*

[a] Individuals seropositive on entry that subsequently became seronegative.
[b] Individuals seronegative on entry that subsequently became seropositive.
[c] The second bleed was at 30 days post-entry except for three flagged individuals whose second bleed was at 60 days.

Table 4.9: Serial reciprocal titres of ten serologically monitored flying foxes whose neutralising antibody titres to HeV showed a fourfold or greater fluctuation over a minimum 12 month period.

Date	Flying fox microchip number									
	0001 232432	0001 3204A0	0001 3C709A	0001 34EEA6	0001 34F4DB	0001 D38B13	0001 DB945F	0001 DB0B25	0001 DB05DC	0001 DAD405
Jan-97		160						80		
Feb-97	40	160	80	10	10	5	20	80	10	40
Mar-97		>160			10		80	>160	>160	80
Apr-97	>160	>160	>160	>160	10	10	>160	>160	80	80
May-97	320	>320	320	80	20	20	>320	>320	160	160
Jun-97	>320			160	40				20	
Jul-97	>640		320	320	80	160			80	
Aug-97	>640	>640	>640	160	160	160	>640	320	160	160
Sep-97	>640	>640	320	160	80	160			160	80
Oct-97	>640	160	160	160	80	160	160	320	80	80
Nov-97	>640	>640	80	40	80	40	160	>640	80	160
Dec-97	160	>640	80	80	20	20	320	>640	80	160
Jan-98										
Feb-98	40	80	40	40	5	5	80	160	20	80
Mar-98	40	40	40	80	20	<5	40	40	80	40
Apr-98	320	320	80	80	40	40	80	320	80	80
May-98	>40			>40	>40	>40	>40			
Jun-98	160			40	40	10	160			
Jul-98	320			80	10	40	320			
Aug-98	320			40	40	40	320			

Table 4.10: Serial reciprocal titres of one serologically monitored flying fox whose neutralising antibody titres to HeV remained static[1] over a minimum 12-month period.

Date	Flying fox microchip number
	0001DC4051
Jan-97	320
Feb-97	
Mar-97	>160
Apr-97	>160
May-97	>320
Jun-97	
Jul-97	
Aug-97	>640
Sep-97	>640
Oct-97	>640
Nov-97	>640
Dec-97	>640
Jan-98	
Feb-98	>640
Mar-98	>320
Apr-98	>640

[1] Titres did not exhibit a fourfold or greater change.

Table 4.11: Serial reciprocal titres of nine serologically monitored flying foxes whose neutralising antibody titres to HeV fell over a minimum 12-month period.

Date	Flying fox microchip number							
	0001 216DF7	0001 DB044F	0001 FB41CA	4105 4D7E52	4105 5A0437	4106 5C567C	4105 4C4E3B	0001 DC4B7A
Jan-97		5						
Feb-97	<5	<5	40					40
Mar-97	10	<5						40
Apr-97	<5	5	5					80
May-97	5	<5	20					80
Jun-97	5	<5	<5					40
Jul-97	<5	<5	20					
Aug-97	<5	<5	10					80
Sep-97	5	<5	5					40
Oct-97	<5	<5	<5					40
Nov-97	<5	<5	<5	320	<5	<5	>640	40
Dec-97	<5	<5	<5	160	320	40	80	5
Jan-98			<5					
Feb-98			<5	80	40	10	5	5
Mar-98			<5	80	80	5	20	10
Apr-98			<5	80	160	5	20	5
May-98			<5	>40	80	80	>40	>40
Jun-98			<5	80	80	0	5	<5
Jul-98			<5	40	40	5	5	<5
Aug-98				20	20	0	<5	<5
Sep-98				80	80	40	40	
Oct-98				40	40	0	80	5
Nov-98				<40	10	5	<5	
Dec-98				40	20	5	5	

4.4 Discussion

Study 1

The colony originated from flying foxes taken from the wild in the mid-1980s. It was augmented over the years by breeding, introductions of rehabilitated sick and injured wild flying foxes and surplus flying foxes from other captive colonies. Incomplete records prevented calculation of the proportion of flying foxes from each source and archived sera were not available. Thus, it was not possible to determine whether the observed 19% crude seroprevalence represented infection prior to entry to the colony or whether

infection was circulating in the colony. Assuming a maximum longevity of 15 years (McIllwee & Martin, 2002), few of the original wild-caught flying foxes were likely to remain in the colony. Species-specific seroprevalence in this captive colony (*P. alecto* 31%, 95% CI 9-61%; *P. poliocephalus* 16%, 95% CI 8-29%) broadly parallels that reported in free-living flying foxes in Chapter 3.

Study 2

The isolation of virus implies that transmission can occur in flying foxes in a captive environment. The absence of detected infection in any other enrolled flying fox suggests that the source of infection was either an unenrolled captive flying fox in the enclosure or, as the colony was not effectively closed, a free-living flying fox which effected transmission across the woven wire enclosure barrier. The former is initially attractive, given that, of the 100 flying foxes in the colony, only 17 were enrolled in this study. However, on reflection, the occurrence of a single isolated infection in a predominantly seronegative (15/17) study cohort challenges this interpretation, moreso given that the infection was detected in the 8th week of a 14-week study. An alternate interpretation is that the isolated 'case' resulted from recrudescence of a latent infection, rather than as a result of horizontal transmission within or from outside the colony. Three observations make this latter scenario plausible: the absence of detectable infection in any of the other fourteen seronegative in-contact flying foxes enrolled in the study; the apparent absence of virus excretion in the infected animal; and (less so) the absence of neutralising antibodies in the infected animal post-infection. Standard infectious disease theory argues that, given effective exposure of susceptible animals (so-called adequate contact[20]), transmission will occur and infection will spread through a population (Bailey, 1975). While the incubation period of HeV in flying foxes remains to be defined, experimental studies have recovered virus at 10 days post-inoculation but not at 21 days post-inoculation (Williamson *et al.*, 2000). Thus the continuation of this study for a further 42 days after the isolation represents a reasonable timeframe for evidence of infection to appear in the in-contact flying foxes, particularly given that this is a captive population, and that the number of contacts per unit time is likely to be exaggerated. While it could be argued that the apparent absence of virus in throat or urogenital swabs is not evidence of absence of excretion, particularly when the portal of exit of HeV has yet to be demonstrated in flying foxes, other studies support urine and saliva as the most

[20] Adequate contact has three components: the number of contacts per unit time, the transmission potential per contact, and the duration of infectiousness.

likely routes of excretion for HeV (Williamson *et al.*, 1998) and the closely related Nipah virus (Chua *et al.*, 2002). Both routes were monitored in this study. Further, the sampling interval of seven days was sufficiently short to have a high likelihood of detecting even a short-lived excretion. The significance of the lack of neutralising antibodies in the infected animal post-infection is less clear. Given the reconfirmation of the presence of virus in the original sample by cell culture and by PCR, there are two plausible interpretations for the lack of neutralising antibodies post-infection: either the viraemic animal failed to seroconvert, or the animal was not monitored for long enough to detect seroconversion. In support of the former, there is experimental evidence for absence of seroconversion in susceptible flying foxes inoculated with an infective dose of HeV. Two of four flying foxes inoculated subcutaneously and one of four inoculated orally failed to generate detectable neutralising antibody response by 21 days post-inoculation (Williamson *et al.*, 1998). Unfortunately the viraemic status of the animals was not recorded. In the same study, the remaining two of four flying foxes inoculated subcutaneously and three of four inoculated parenterally seroconverted within 21 days post-inoculation (Williamson *et al.*, 1998). The flying fox in this study was monitored for only 14 days post-isolation (at which time it was euthanased[21]), thus the possibility of seroconversion between days 14 and 21 cannot be excluded. Interpretation of the absence of virus on necropsy is challenging. While virus isolation by cell culture can lack sensitivity, detection of viral genome by RT-PCR is generally regarded as highly sensitive, even with latent virus. Further, some latent viruses (herpesviruses and retroviruses) are actually reactivated by cell culture. Thus, given the confirmed presence of virus in the original sample, the negative findings at necropsy suggest that the site of viral latency was not sampled.

Study 3

The strong association between Species and HeV serostatus on entry (Table 4.5) reflects the purposive sampling methodology. Only eight spectacled flying foxes were enrolled in the study, all of them seropositive on entry.

There was a strong association (RR=9, 95% CI 1.42, 57.12) between dam serostatus and pup serostatus. When the two years of data were combined, pups born to seropositive

[21] The timing of the euthanasia was dictated by mounting Workplace Health and Safety concerns.

dams were nine times more likely to be seropositive at their second bleed[22] than were pups born to seronegative dams. While sparseness of data precluded meaningful analysis of the separate 1996-97 and 1997-98 season, the trend for each suggests the same association. Interpretation of the association is not straightforward, with plausible arguments for both vertical transmission and for maternal antibody transfer. The latter is supported by the lack of seropositive pups from seronegative dams. Only one of eight pups born to seronegative dams was seropositive on entry. If flying foxes can be latently infected in the absence of seroconversion, as argued in relation to the viraemic animal in Study 2, and assuming resultant vertically infected pups become seropositive, then the association between seropositive pups and seropositive dams might be expected to be weaker. That said, the 95% confidence interval is wide (reflecting the smaller sample size) and over-emphasis of the point value is unwise. The argument for vertical transmission is supported by several observations. Firstly, three of the eight seropositive pups born to seropositive dams were seronegative at their first bleed. Further, given that pup age at entry ranged from 2 to 6 weeks, it is possible that some of the five that were seropositive on entry were seronegative earlier. Secondly, the persistence of passively acquired maternal antibodies beyond six months (as was the case with five of the eight pups) is improbable, although specific data on flying foxes is lacking. Unfortunately, an inability to identify the immunoglobulin class precluded conclusive identification of the origin of the antibodies in pups.

Three flying foxes seroconverted during the study period. All were pups born in the 1996-97 season (Table 4.6). The absence of seroconversion in any of the other 32 flying foxes that were seronegative at entry argues against horizontal transmission of infection, and the two observations together better support a hypothesis of vertical transmission. The only other change in serostatus in the study was in three individuals that were seropositive on entry, but seronegative at subsequent bleeds (Table 4.7). The first was a mature male (0001DB044F) monitored from January to December 1997 who had titres of <1:5 for all but the first (1:5) and fourth (1:5) bleeds. Twofold changes in serial titres are generally not considered significant (Thrusfield, 1986), and it is probable that this individual was truly seronegative throughout the study. The second, a 1996-97 season pup (0001FB41CA) born to a seropositive dam and monitored from February 1997 to October 1998, had consecutive monthly serial titres of 1:40, na, 1:5, 1:20, <1:5, 1:20, 1:10 and 1:5, followed

[22] The second bleed was chosen because it had the least missing data, although, at 30-60 days post-entry, it was recognised that it could include passively acquired and actively acquired antibodies.

by titres of <1:5 until censored. As previously argued, this duration of antibody persistence is more consistent with active infection than passive maternal antibody transfer, thus the more plausible interpretation is that this individual was truly seropositive at or soon after birth, and that the immunity decayed over a seven month period. Antibody persistence in pups is discussed further in the next paragraph. The third, a 1997-98 pup (410666530E) had a titre of 1:160 on entry and a titre of <1:5 at the second bleed 30 days later. The pup's dam was seronegative throughout the study, so the initial titre cannot be attributed to maternal antibody transmission. This leaves two possible alternatives: the initial titre resulted from active infection, or it was a false result due to misidentification. The former seems improbable for two reasons: firstly, none of 7 other pups born to seronegative dams showed antibody titres at any time in the study; and secondly, a seven-fold drop in titre in a 30 day period is improbable. The alternative is that the sample was misidentified, either in the field, in the laboratory, or at reporting. Unfortunately the pup was lost to follow-up after the second bleed.

Ten of nineteen flying foxes monitored for twelve months or more exhibited a fourfold or greater movement in neutralising antibody titres over that period (Table 4.9). There are two possible interpretations of this observation: either the movement is real or a reflection of poor test repeatability. Given the technical expertise and the quality assurance systems at AAHL[23], the latter seems improbable. Also, if the change were due to poor test repeatability, it might reasonably be expected across all 19 flying foxes. Certainly Thrusfield (1986) comments that geometric fourfold changes are generally regarded as reflecting real change. The apparent temporal pattern of titre movements (titres rising in the second half of the year) further supports the movements being real, and suggests a seasonal challenge to the immune system that boosts antibody titre. Eight of the ten were mature females; the other two were mature males. A single mature male flying fox whose titres varied between >1:160 and >1:640 was classified as having static titres (less than a fourfold change over the study period). The lack of absolute values for this animal precluded calculation of definitive titre movement. The remaining eight of the 19 flying foxes had falling titres (Table 4.11). Seven were pups. Three of the eight whose titres fell to <1:5 (that is, became seronegative) were discussed in the previous paragraph. The titres of the remaining five (all pups) trended lower over the observation period and presumably would have become seronegative given a longer observation

[23] AAHL has NATA accreditation and is Australia's peak animal health laboratory.

period. The length of time over which these titres decayed is more consistent with a waning primary antibody response to a challenge in the absence of a subsequent 'booster' challenge.

General discussion

A number of findings suggest that vertical transmission of Hendra virus infection is occurring in this captive population of flying foxes. Experimental studies support the possibility of vertical transmission of Hendra virus. Experimental infections in pregnant guinea pigs and flying foxes indicate a predilection of HeV for the reproductive tract (Williamson *et al.*, 2000), with virus recovered from the uterus and placenta of ten of eleven pregnant guinea pigs, and virus antigen detected in ovarian and foetal tissues. Viral titres in pregnant guinea pigs were generally higher than in non-pregnant guinea pigs. In experimentally infected pregnant flying foxes, the recovery of virus from foetal tissues, and positive immunostaining of placental veins demonstrated the capability for transplacental transmission. No virus was recovered from the mouth, nose, rectum or urine of these animals.

A plausible mechanism of vertical transmission in flying foxes involves recrudescence of latent infection in the pregnant female, and transmission to the in-utero or neonate pup, where a reduced immune response allows persistence of the virus. The concepts of persistence[24] and latency[25] are well recognized. The human polyomaviruses represent a useful analogy to the hypothesis proposed for HeV. These viruses remain latent in the kidney of healthy adults until reactivated and shed in urine[26] during pregnancy, in old age or in immunosuppressed states (Mims *et al.*, 1995). Where infection occurs in-utero or in early post-natal life, a degree of immunological tolerance to the agent is common. This tolerance can manifest as a weak immune response that is unable to control infection, or where in-utero infection occurs during the development of the immune system, can incur no immune response whatsoever because the agent's antigens are regarded as 'self' antigens (Mims *et al.*, 1995). Latency and recrudescence of Hendra virus infection

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[24] Persistence is a state where the agent is continuously present in a clinically normal host. Shedding is more or less continuous (Mims *et al*, 1995).

[25] Latency represents an extreme manifestation of persistence whereby the agent is present but inapparent in the host until activated by certain triggers (Mims *et al*, 1995).

[26] That many persistent viruses are shed via urine, saliva or milk can be explained by the frequent inaccessibility of lumenal cells (in the kidney tubule, salivary gland or mammary gland) to an effective host immune response.

associated with pregnancy is also consistent with the previously advanced hypothesis that spillover from flying foxes to horses is effected by contact with infected foetal tissues or fluids, via the ingestion of recently contaminated pasture (Field *et al.*, 2000; Halpin *et al.*, 2000; Young *et al.*, 1997). Further, evidence of latency and recrudescence was seen in the second human Hendra virus case, when a fulminating infection occurring 13 months after the original infection (O'Sullivan *et al.*, 1997). This case also evidenced the ability of the virus to persist in the presence of neutralising antibodies.

It is not the intent of the preceding discussion to argue vertical transmission as the sole or the primary mode of transmission of Hendra virus in flying foxes, but rather to critically discuss the study findings and present biologically plausible interpretations. It should be noted that this study population was captive, and that not only the dynamics of infection, but also the significance of any vertical transmission may be very different in free-living population. The presence of an association between age and HeV serostatus and absence of an association between sex and HeV serostatus in free-living flying foxes (Chapter 3) argue against vertical transmission as the primary mode of transmission of Hendra virus, as does the finding of persistent antibody titres in mature male flying foxes in this study. Further, experimental studies in horses and cats have demonstrated Hendra virus in urine and saliva (Williamson *et al.*, 1998), and experimental (Daniels *et al.*, 2001b) and observational (Chua *et al.*, 2002) studies of the closely related Nipah virus in flying foxes have recovered virus from urine and partially eaten fruit. Thus, it is evident that further studies (observational, experimental and theoretical) are necessary to fully understand this fundamental aspect of the ecology of Hendra virus.

4.5 Conclusions

The positive serological findings of Study 1 demonstrated evidence of Hendra virus infection in the captive study population, but not whether exposure was recent or historic. Studies 2 and 3 suggest the presence of current infection in the colony, the former by virus isolation, the latter based on seroepidemiology. The isolation of virus from a single mature female flying fox in Study 2 better fits recrudescence of a latent infection rather than horizontal transmission. Similarly, in Study 3, the statistical association between dam and pup serostatus, the persistence of antibodies in pups, and the seasonal boost in mature female titres are more consistent with vertical transmission. A plausible mechanism of vertical transmission in flying foxes is proposed, involving recrudescence of latent infection in the pregnant female, and transmission to the in-utero or neonate pup,

where a reduced immune response allows persistence of the virus. The hypothesis is supported by experimental studies in pregnant animal models that suggest Hendra virus affinity for the reproductive tract and the capacity for transplacental transmission.

The role of vertical transmission is unclear, and further research is needed to fully understand the dynamics of Hendra virus infection in flying foxes. Research priorities include longitudinal observational studies to investigate temporal patterns of infection and routes of excretion in flying foxes, experimental studies focusing on infection dynamics in the pregnant flying fox, and the origin and persistence of antibodies in pups, and modelling studies to identify key parameters determining the rate of infection and persistence of infection in flying fox populations. The latter is addressed in Chapter 6.

CHAPTER FIVE

INVESTIGATIONS OF THE ECOLOGY OF AUSTRALIAN BAT LYSSAVIRUS

5.1 Introduction

The intensity of surveillance of Australian bats increased after flying foxes were identified as a natural host and probable reservoir of Hendra virus (see Chapter 3) as researchers sought to elaborate the ecology of the new virus. In May 1996, just months later, a previously unknown lyssavirus (subsequently named Australian bat lyssavirus) was identified in a black flying fox (*Pteropus alecto*) in northern New South Wales. Australia had previously been considered free of lyssaviral infections. Notwithstanding marked antigenic and genetic similarities to rabies virus, Australian bat lyssavirus is phylogenetically distinct, and represents a new lyssavirus genotype (genotype 7) (Gould *et al.*, 1998; Hooper *et al.*, 1997).

Within six months of the identification of ABLV in flying foxes, the zoonotic capability of the virus became evident when a wildlife carer in central Queensland developed a fatal rabies-like illness in October 1996. A second human death occurred in December 1998, twenty-seven months after a bite from a flying fox (Allworth *et al.*, 1996; Hanna *et al.*, 2000).

Was ABLV a recent introduction to Australian bat populations? Had previous cases in humans or animals been misdiagnosed? How widespread was the infection in flying foxes? Were other bat species infected? A Lyssavirus Expert Group meeting in late 1996 identified a number of research priorities for ABLV. The DPI&F undertook investigations relating to several of these: a retrospective study of animal encephalitides, the pathological examination of sick bats and bats with a history of biting people, and the frequency and distribution of ABLV in Australian bat populations. This chapter represents the latter investigations. Section 5.2 describes the investigation of the role of flying foxes, Section 5.3, the role of insectivorous species, and Section 5.4, a retrospective investigation of archived bat specimens.

5.2 THE OCCURRENCE OF AUSTRALIAN BAT LYSSAVIRUS IN FLYING FOXES (SUB-ORDER MEGACHIROPTERA).

5.2.1 Introduction

As previously stated, four species of flying fox occur on mainland Australia. Their ranges frequently overlap, resulting in different species sharing food and roosting resources. Most species are highly mobile, with populations and camps frequently in a state of flux as animals respond to food availability and breeding behavior. In urban areas, flying fox numbers appear to be increasing, a phenomenon attributed to disruption of natural food sources as a result of continuing habitat loss. Thus the discovery of the rabies-like ABLV in flying foxes raised not only livestock trade concerns, but serious public health concerns. Against this background, this study sought to establish the distribution and frequency of ABLV in Australian flying fox populations.

The primary objectives of this study were to

1. Identify the geographic and species distribution of ABLV infection in Australian flying foxes;
2. Estimate the prevalence of ABLV infection in flying fox populations;
3. Identify risk factors for infection in flying foxes.

5.2.2 Materials and methods

5.2.2.1 Study design

The study took the form of a cross-sectional survey of non-randomly (purposively) selected flying fox populations on mainland Australia. The study population comprised the known accessible flying fox colonies in each surveyed State, and flying foxes coming into human care in Queensland and New South Wales. The individual was the unit of interest. Either brain samples or blood and brain samples were taken from each individual.

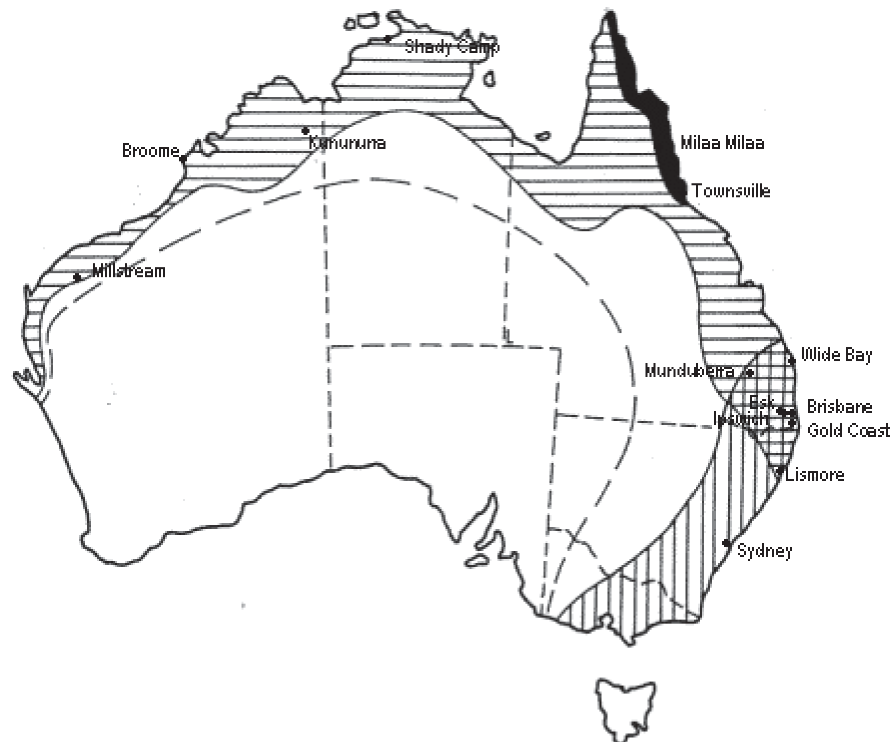
5.2.2.2 Sampling methodology

Flying foxes were non-randomly sampled by one of two methodologies - wild-caught or opportunistic. Wild-caught specimens were collected by mist-net (Queensland, Northern Territory) and by shooting (Western Australia). These methodologies are described in detail in Chapter 3 (3.3.2.3). The opportunistic sample consisted of sick, injured or recently dead flying foxes submitted as diagnostic specimens to YVL by wildlife authorities, wildlife rescue groups, or members of the general public.

5.2.2.3 Sampling locations

Wild-caught flying foxes were collected from multiple locations in Queensland, New South Wales, the Northern Territory, and Western Australia. Opportunistic specimens were obtained from wildlife authorities, carers, and institutions in the greater Sydney area in New South Wales, and from the greater Brisbane area, the Gold Coast, Wide Bay and the Atherton Tableland in Queensland. See Figure 5.1.

Figure 5.1: Distribution of flying foxes on mainland Australia[1] and sampling locations of wild-caught and opportunistic specimens.



Key: Horizontal hatching = *P. alecto*
Vertical hatching = *P. poliocephalus*
Solid black = *P. conspicillatus*
Broken line = southern inland limit of *P. scapulatus*

[1] Adapted from Hall and Richards (2000).

5.2.2.4 Sample size

For wild-caught flying foxes, a minimum sample size of 60 individuals per species per location was sought, enabling detection of antigen or antibody (with 95% statistical confidence) at a minimum population prevalence of 5%, assuming 100% test sensitivity and specificity. Opportunistic specimens were received on an ad-hoc basis.

5.2.2.5 Sampling period

The sampling period in Queensland was April 1996 to October 2002; in New South Wales, November 1996 to October 1998; in the Northern Territory, August 1999 and March 2001; and in Western Australia, December 1998, February 2001 and June 2001.

5.2.2.6 Specimens collection

Initially, brain was the primary specimen sought (for antigen detection studies). Typically, the euthanased flying fox was decapitated, the temporal muscle dissected, and the cranium removed with bone cutters and/or scissors. The dura was incised and reflected and the brain removed. One hemisphere was placed in 10% formalin and the other refrigerated pending FAT, after which it was frozen at -70°C. Where there was temporal overlap with Hendra virus research, a ranges of tissues was also collected for HeV isolation attempts[27]. Later blood (for antibody studies) and brain samples were collected from each flying fox.

5.2.2.7 Laboratory methodologies

Fresh brain impression smears were tested for ABLV antigen at YVL using a fluorescent antibody test incorporating an anti-rabies antibody. The smears included tissue from at least three sites (medulla, cerebellum and hippocampus) on the cut brain surface. The detailed methodologies for producing smears and for performing the FAT are described in the YVL *Laboratory Methods Manual* (Volume 2). Sera were forwarded to AAHL for testing for the presence of neutralising antibodies[28] by RFFIT. For each dilution, a reduction of 50% or more in the number of fields with fluorescing cells (compared to the antibody negative controls)

[27] Specimens collected between 1996 and 1998 provided tissue samples for concurrent Hendra virus (Chapter 3) and ABLV studies.

[28] The initial RFFIT used by AAHL employed a rabies virus antigen. ABLV and classical rabies virus both belong to lyssavirus serotype 1(Fraser *et al*, 1996) and consequently cross-neutralise. Given the absence of classical rabies or other serotype 1 lyssaviruses in Australia, the rabies-based RFFIT provided a practical screening test for ABLV while a specific ABLV RFFIT was being developed. AAHL later used rabies virus antigen and specific ABLV antigen RFFITs in parallel.

was interpreted as indicating the presence of neutralising antibodies. The detailed test methodology used by AAHL is as reported by Smith *et al* (1973).

5.2.2.8 Data management and statistical analysis

Data were recorded on outcomes variables *FAT status* and *RFFIT status*, and on six possible explanatory variables: *species*, *sex*, *age*, *sampling location*, *sampling year*, and *sampling method*. *Age* class (immature, mature) was determined on the basis of physiological criteria indicating sexual maturity, and included weight and forearm length, dentition wear, and mammary development. All data were stored and managed in a Microsoft ™ Access 97 ™ database. Data was exported to Microsoft ™ Excel 97 ™ for descriptive and univariate analyses. EpiInfo (Version 6) Statcalc was used in the latter to test for associations between each of the outcome variables and the possible explanatory variables, with Chi square *p* values indicating the statistical significance of associations, and unadjusted relative risk (RR) indicating the magnitude of associations[29]. Yates Corrected *p* value was used except where cell values were less than 5, when Fisher's Exact *p* value was used. Stratified analysis was used to detect confounding and/or interaction between the possible explanatory variables[30]. A Kappa test was used to evaluate agreement between the FAT and the RFFIT in the group that had both brain and blood screened. In addition to the kappa statistic that measures the overall level of agreement between the tests, two separate indices of proportionate agreement, P (positive) and P (negative), are also used as proposed by Cicchetti and Feinstein (1990) and Feinstein & Cicchetti (1990). P (positive) and P (negative) are analogous to sensitivity and specificity for concordance.

5.2.3 Results

A total of 1477 flying foxes from all four mainland Australian species and from locations in Queensland, New South Wales, the Northern Territory and Western Australia was surveyed over a five year period. Samples collected from each individual consisted of either brain and blood (281), or brain only (1196). Of the *brain and blood* group (Table 5.1), 7 (2.5%)

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[29] As in Chapter 4, relative risk was used in preference to odds ratio because the former is more readily and widely interpretable, and because stratified analysis does not mandate the use of odds ratios.

[30] Stratification was used in preference to logistic regression because of the small number of possible explanatory variables, and the ability of the former approach to clearly and concisely demonstrate effect modification.

were positive for antigen, and 12 (4.3%) were positive for antibody, with 2 individuals being positive for both antigen and antibody. These individuals were a mature male opportunistic *P. alecto* sampled in Queensland in 1997 and a mature male opportunistic *P. poliocephalus* sampled in Queensland in 1998.

Table 5.1: Characteristics of 281 non-randomly sampled flying-foxes from Queensland, the Northern Territory and Western Australia screened for ABLV by both FAT and RFFIT between April 1996 and June 2001.

Variable	Number of flying foxes tested		
	Total	Positive by	
		FAT	RFFIT
Species			
P. scapulatus	119	1	9
P. poliocephalus	2	2	1[1]
P. alecto	160	4	2
Sex			
Male	150	3	6
Female	121	4	6[1]
Unknown	10	0	0
Age			
Immature	75	0	4
Mature	196	7	7[1]
Aged	1	0	0
Unknown	9	0	1
SampleMethod			
Wild-caught	266	0	8
Opportunistic	15	7	4[1]
SampleLocation			
NT	89	0	4
Qld	15	7	4
WA	177	0	4
SampleYear			
1996	8	0	2
1997	5	5	1[1]
1998	116	2	2
1999	50	0	0
2001	102	0	7
Total	281	7	12

[1] RFFIT result unknown for one individual

One of the seven antigen-positive individuals had an unknown antibody status. The kappa value measuring the overall level of agreement between the standard FAT (antigen) test and the new RFFIT (antibody) test was 0.18 (Figure 5.2). The P (positive) value was 0.21 and the P (negative) 0.97. There was substantial asymmetry along the concordant diagonal.

Figure 5.2: Level of agreement (Kappa) between FAT and RFFIT on 280[1] flying foxes screened for both antigen and antibody.

		FAT	
		Positive	Negative
RFFIT	Positive	2	10
	Negative	4	264

% agreement: 95%
Kappa: 0.18
P(pos): 0.21
P(neg): 0.97

[1] Seven of the 281 flying foxes in the *blood and brain* group had a positive FAT result, however one of the seven had an unknown RFFIT result and was excluded from the Kappa analysis.

Of the *brain only* group (Table 5.2), 62 (5.2%) were positive for antigen. All antigen-positive individuals were opportunistic specimens. Of the total 902 opportunistic specimens screened for antigen, 69 (8.3%) were positive for antigen (Table 5.3). Of the 281 flying foxes screened for antibody, 280 returned results. Antibody was detected in 4/14 (28.6%) opportunistic specimens and in 8/266 (3%) wild-caught individuals (Table 5.4). ABLV antigen or antibody was detected in flying foxes in Queensland, Western Australia and the Northern Territory.

Table 5.2: Characteristics of 1096 non-randomly sampled flying-foxes from Queensland, New South Wales and Western Australia screened for ABLV by FAT only between April 1996 and October 2002.

Variable	Number of flying foxes tested	
	Total	Positive by FAT
Species		
P. scapulatus	191	21
P. poliocephalus	269	7
P. alecto	541	33
P. conspicillatus	95	1
Sex		
Male	489	30
Female	500	26
Unknown	107	6
Age		
Immature	352	12
Mature	585	41
Aged	43	0
Unknown	116	9
SampleMethod		
Wild-caught	209	0
Opportunistic	887	62
SampleLocation		
Qld	1044	62
WA	21	0
NSW	31	0
SampleYear		
1996	171	4
1997	369	17
1998	299	20
1999	102	4
2000	102	9
2001	37	5
2002	16	3
Total	1096	62

Table 5.3: Univariate association between a number of independent variables and FAT status in 1377[1] non-randomly sampled flying foxes from Queensland, the Northern Territory and Western Australia surveyed for ABLV between April 1996 and June 2001.

Variable	Number of flying foxes testing		P value	Relative Risk (95% CI)
	FAT positive	FAT negative		
Species			0.05	
P. conspicillatus	1	94		Referent
P. poliocephalus	9	262		3.15 (0.41, 24.58)
P. alecto	37	664		5.01 (0.7, 36.13)
P. scapulatus	22	288		6.74 (0.92, 49.36)
Sex[2]			0.79	
Female	30	591		Referent
Male	33	606		1.07 (0.66, 1.73)
Age[3]			0.02	
Immature	12	415		Referent
Mature	48	777		2.07 (1.11, 3.86)
SampleMethod			<0.001	
Wild-caught	0	475		
Opportunistic	69	833		Unable to be calculated[4]
SampleLocation			<0.001	
WA & NT[5]	0	318		
Qld & NSW[5]	69	990		Unable to be calculated[6]
SampleYear			0.89	
1996-98	48	920		Referent
1999-02	21	388		1.04 (0.63, 1.71)

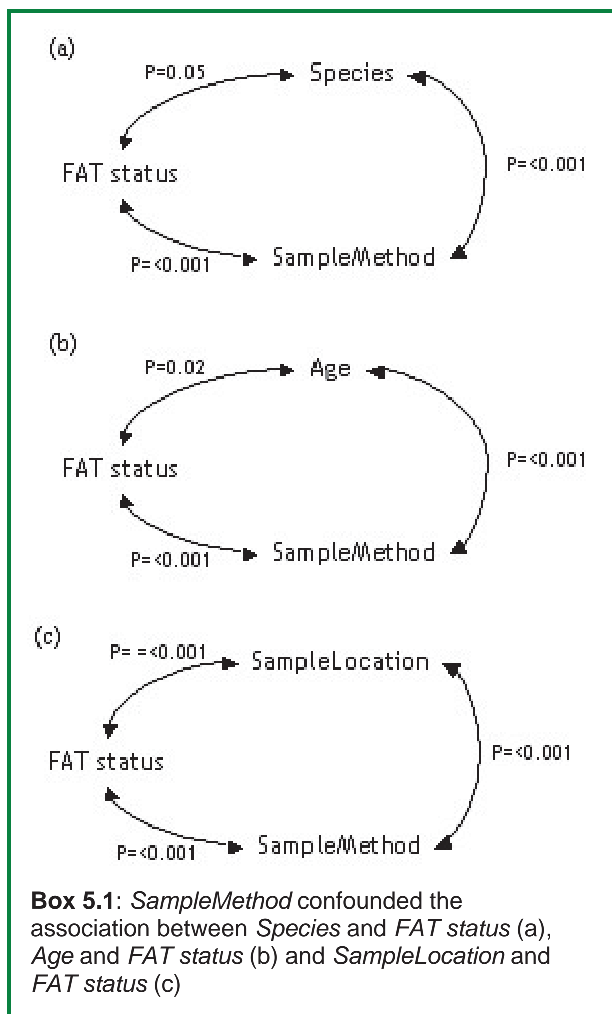
[1] Combined data from Tables 5.1 and 5.2. Where biologically sensible, data were collapsed into two strata per explanatory variable to facilitate and simplify analysis.
[2] 117 individuals of unknown sex excluded from the analysis.
[3] 125 individuals of unknown age excluded from the analysis, and 44 *Aged* individuals combined with *Mature.*
[4] The zero positive value precludes calculation, but for illustration, if 0 is replaced by 1, the relative risk is 36.31 (5.07, 261.31).
[5] The data were grouped in this manner to reflect the accepted regional population structure.
[6] The zero positive value precludes calculation, but for illustration, if 0 is replaced by 1, the relative risk is 20.78 (2.9, 149.07).

Table 5.4: Univariate association between a number of independent variables and RFFIT status in 279[1] non-randomly sampled flying-foxes from Queensland, the Northern Territory and Western Australia surveyed for ABLV between April 1996 and June 2001.

Variable	Number of flying foxes testing		P value	Relative Risk (95%CI)
	RFFIT positive	RFFIT Negative		
Species[2]			0.01	
P. alecto	2	158		Referent
P. scapulatus	9	110		6.05 (1.32, 27.49)
Sex[2]			0.93	
Male	6	144		Referent
Female	6	115		1.24 (0.41, 3.75)
Age[3]			0.5	
Mature	7	190		Referent
Immature	4	71		1.5 (0.45, 4.98)
SampleMethod			0.002	
Wild-caught	8	258		Referent
Opportunistic	4	10		9.5 (3.25, 27.78)
SampleLocation			0.002	
WA & NT[4]	8	258		Referent
Qld	4	10		9.5 (3.25, 27.78)
SampleYear[5]			0.76	
1996-98	5	123		Referent
1999-01	7	145		1.18 (0.38, 3.63)

[1] Data from Table 5.1 minus one *P. poliocephalus* of unknown serostatus and the sole remaining *P. poliocephalus.* Where biologically sensible, data were collapsed into two strata per explanatory variable to facilitate and simplify analysis.
[2] Ten individuals of unknown sex were excluded from the analysis.
[3] Nine individuals of unknown age were excluded from the analysis, and the sole *Aged* individual combined in *Mature.*
[4] The data were grouped in this manner to reflect the accepted regional population structure.
[5] Years 1996-1998 and 1999-2001 data combined.

Univariate analysis of a combined dataset of all flying foxes screened by FAT[31] identified a statistical association between *FAT status* and the explanatory variables *Species* (<0.001), *Age* (0.02), *SampleMethod* (<0.001) and *SampleLocation* (<0.001) (Table 5.3). However, a series of additional univariate analyses showed *SampleMethod* was confounding the association of *Species* (Table 5.5), *Age* (Table 5.6), and *SampleLocation* (Table 5.7) with *FAT status* (see Box 5.1 also). When *Species* was stratified by *SampleMethod* (Table 5.8), the association between *Species* and *FAT status* in opportunistically sampled flying foxes was strengthened. Zero values in the *Wild-caught* stratum precluded identification of any possible interaction. When *Age* was stratified by *SampleMethod* (Table 5.9), the association between *Age* and *FAT status* was also strengthened in opportunistically sampled flying foxes. Zero values in the *Wild-caught* stratum again precluded identification of any possible interaction. When *SampleLocation* was stratified by *SampleMethod* (Table 5.10), zero values precluded further analysis.



Box 5.1: *SampleMethod* confounded the association between *Species* and *FAT status* (a), *Age* and *FAT status* (b) and *SampleLocation* and *FAT status* (c)

[31] That is, flying foxes from both the *brain and blood* group and the *brain only* group.

Table 5.5: Association between the explanatory variables *Species* and *SampleMethod* in 1377[1] non-randomly sampled flying-foxes from Queensland, the Northern Territory and Western Australia screened for ABLV by FAT between April 1996 and June 2001.

	SampleMethod		P value	Relative Risk (95% CI)
	Opportunistic	Wild-caught		
Species			<0.001	
P. scapulatus	126	184		Referent
P. alecto	481	220		1.69 (1.46, 1.95)
P. poliocephalus	200	71		1.82 (1.56, 2.11)
P. conspicillatus	95	0		2.46 (2.15, 2.81)

[1] Table 5.3 data

Table 5.6: Association between the independent variables *Age* and *SampleMethod* in 1252[1] non-randomly sampled flying foxes from Queensland, the Northern Territory and Western Australia screened for ABLV by FAT between April 1996 and June 2001.

	SampleMethod		P value	Relative Risk (95% CI)
	Opportunistic	Wild-caught		
Age			<0.001	
Mature	489	336		Referent
Immature	297	130		1.17 (1.08, 1.28)

[1] Table 5.3 data and excludes 125 individuals of unknown age.

Table 5.7: Association between the independent variables *SampleLocation* and *SampleMethod* in 1377[1] non-randomly sampled flying foxes from Queensland, the Northern Territory and Western Australia screened for ABLV by FAT between April 1996 and June 2001.

	SampleMethod		P value	Relative Risk (95% CI)
	Opportunistic	Wild-caught		
SampleLocation			<0.001	
WA & NT	0	318		
Qld & NSW	902	157		Unable to be calculated[2]

[1] Table 5.3 data
[2] The zero values precludes calculation.

Table 5.8: Association between the explanatory variable *Species* and *FAT status* when stratified by *SampleMethod* in 1377[1] non-randomly sampled flying-foxes from Queensland, the Northern Territory and Western Australia screened for ABLV by FAT between April 1996 and June 2001.

	FAT status		P value	Relative Risk 95%CI)
	Positive	Negative		
Opportunistic			<0.001	
P. conspicillatus	1	94		Referent
P. poliocephalus	9	191		4.28 (0.55, 33.26)
P. alecto	37	444		7.31 (1.01, 52.62)
P. scapulatus	22	104		16.59 (2.28, 120.89)
Wild-caught			Unable to be calculated[2]	
P. conspicillatus	0	0	Unable to be calculated[2]	Unable to be calculated[2]
P. poliocephalus	0	71	Unable to be calculated[2]	Unable to be calculated[2]
P. alecto	0	220	Unable to be calculated[2]	Unable to be calculated[2]
P. scapulatus	0	184	Unable to be calculated[2]	Unable to be calculated[2]

[1] Table 5.3 data.
[2] The zero values precludes calculation.

Table 5.9: Association between the independent variable *Age* and *FAT status* when stratified by *SampleMethod* in 1252[1] non-randomly sampled flying foxes from Queensland, the Northern Territory and Western Australia screened for ABLV by FAT between April 1996 and June 2001.

	FAT status		P value	Relative Risk (95% CI)
	Positive	Negative		
Opportunistic			0.005	
Immature	12	285		Referent
Mature	48	441		2.43 (1.31, 4.5)
Wild-caught			Unable to be calculated[2]	
Immature	0	130		
Mature	0	336		Unable to be calculated[2]

[1] Table 5.3 data and excludes 125 individuals of unknown age.
[2] The zero values preclude calculation, but for illustration, if 0 is replaced by 1, the P value is 0.48 and the relative risk is 0.39 (0.02, 6.17).

Table 5.10: Association between the independent variable *SampleLocation* and *FAT status* when stratified by *SampleMethod* in 1377[1] non-randomly sampled flying foxes from Queensland, the Northern Territory and Western Australia screened for ABLV by FAT between April 1996 and June 2001.

	FAT status		P value	Relative Risk (95% CI)
	Positive	Negative		
Opportunistic			Unable to be calculated[2]	
WA & NT	0	0		
Qld & NSW	69	833		Unable to be calculated[2]
Wild-caught			Unable to be calculated[2]	
WA & NT	0	318		
Qld & NSW	0	157		Unable to be calculated[2]

[1] Table 5.3 data
[2] The zero values preclude calculation.

Univariate analysis of the association between the explanatory variables and *RFFIT status* identified *Species* (*p*=0.01), *SampleMethod* (*p*=0.002) and *SampleLocation* (*p*=0.002) as significant (Table 5.4). The univariate association between *Species* and *SampleMethod* excluded confounding (*p*=0.08, Table 5.11) however, when stratified by *SampleMethod*, the association between *Species* (*P. scapulatus*) and *RFFIT status* was highly significant in the *Wild-caught* stratum and non-significant in the *Opportunistic* stratum, indicating interaction (Table 5.12). The univariate association between *SampleLocation* and *SampleMethod* was highly significant, indicating confounding (Table 5.13). When *SampleLocation* was stratified by *SampleMethod* (Table 5.14), zero values precluded further analysis.

Table 5.11: Association between the independent variables *Species* and *SampleMethod* in 279[1] non-randomly sampled flying foxes from Queensland, the Northern Territory and Western Australia screened for ABLV by RFFIT between April 1996 and June 2001.

	SampleMethod		P value	Relative risk (95% CI)
	Opportunistic	Wild-caught		
Species			0.08	
P. scapulatus	2	117		Referent
P. alecto	11	149		4.09 (0.92, 18.11)

[1] Table 5.4 data

Table 5.12: Association between the independent variable *Species* and *RFFIT status* when stratified by *SampleMethod* in 279[1] non-randomly sampled flying foxes from Queensland, the Northern Territory and Western Australia screened for ABLV by RFFIT between April 1996 and June 2001.

	RFFIT status		P value	Relative Risk (95% CI)
	Positive	Negative		
Opportunistic			0.42	
P. alecto	2	9		Referent
P. scapulatus	1	1		2.75 (0.42, 17.82)
Wild-caught			0.001	
P. alecto	0	149		
P. scapulatus	8	109		Unable to be calculated[2]

[1] Table 5.4 data.
[2] The zero value precludes calculation, but for illustration, if 0 is replaced by 1, the relative risk is 10 (1.3, 80.86).

Table 5.13: Association between the independent variables *SampleLocation* and *SampleMethod* in 280[1] non-randomly sampled flying foxes from Queensland, the Northern Territory and Western Australia screened for ABLV by RFFIT between April 1996 and June 2001.

	SampleMethod		P value	Relative Risk (95% CI)
	Opportunistic	Wild-caught		
SampleLocation			<0.001	
WA & NT	0	266		
Qld	14	0		Unable to be calculated[2]

[1] Table 5.4 data
[2] The zero values preclude calculation.

Table 5.14: Association between the independent variable *SampleLocation* and *RFFIT status* when stratified by *SampleMethod* in 280[1] non-randomly sampled flying foxes from Queensland, the Northern Territory and Western Australia screened for ABLV by RFFIT between April 1996 and June 2001.

	RFFIT status		P value	Relative Risk (95% CI)
	Positive	Negative		
Opportunistic			Unable to be calculated[2]	
WA & NT	0	0		
Qld	4	10		Unable to be calculated[2]
Wild-caught			Unable to be calculated[2]	
WA & NT	8	258		
Qld	0	0		Unable to be calculated[2]

[1] Table 5.4 data.
[2] The zero values preclude calculation.

There was a near-statistically significant univariate association between time of year and FAT status in the 902 opportunistic flying foxes of all species (p=0.067), with those submitted in the first and second quarters being respectively 2.07 (95% CI 1.15 to 3.72) and 1.91 (95% CI 1.0 to 3.63) times more likely to be FAT-positive than those submitted in the referent last quarter (Table 5.15). The association lacked statistical significance when *P. scapulatus* was excluded from the analysis (p=0.29), although flying foxes of the remaining species that were submitted in the second quarter remained 1.96 (95%CI 0.93 to 4.1) times more likely to be FAT-positive than those submitted in the referent last quarter (Table 5.16). When the analysis was repeated on *P. scapulatus* alone, the association was again statistically significant (p=0.054), with those flying foxes submitted in the first quarter being 3.2 (95% CI 1.29 to 7.89) times more likely to be FAT-positive than those submitted in the referent last quarter (Table 5.17).

Table 5.15: Univariate association between time of year and FAT status in 902[1] opportunistic flying foxes of all four species[2] from Queensland and New South Wales[3] surveyed for ABLV between April 1996 and December 2002.

Variable	Number of flying foxes testing		P value	Relative Risk (95% CI)
	FAT positive	FAT negative		
Time of year			0.067	
January-March	19	154		2.07 (1.15, 3.72)
April-June	14	124		1.91 (1.0, 3.63)
July-September	14	163		1.49 (0.78, 2.84)
October-December	22	392		Referent

[1] Data from Table 5.3.
[2] *Pteropus alecto*, *P. poliocephalus*, *P. conspicillatus*, *P. scapulatus*
[3] Only 23 *P. poliocephalus* were from New South Wales; the remaining 879 individuals were from Queensland.

Table 5.16: Univariate association between time of year and FAT status in 776[1] opportunistic flying foxes (*P. alecto*, *P. poliocephalus* and *P. conspicillatus*) from Queensland and New South Wales surveyed for ABLV between April 1996 and December 2002.

Variable	Number of flying foxes testing		P value	Relative Risk (95% CI)
	FAT positive	FAT negative		
Time of year			0.29	
January-March	8	130		1.28 (0.56, 2.92)
April-June	11	113		1.96 (0.93, 4.1)
July-September	12	149		1.64 (0.8, 3.39)
October-December	16	337		Referent

[1] 126 *P. scapulatus* excluded from the analysis.

Table 5.17: Univariate association between time of year and FAT status in 126 opportunistic flying foxes (*P. scapulatus*) from Queensland surveyed for ABLV between April 1996 and December 2002.

Variable	Number of flying foxes testing		P value	Relative Risk (95% CI)
	FAT positive	FAT negative		
Time of year			0.054	
January-March	11	24		3.2 (1.29, 7.89)
April-June	3	11		2.18 (0.62, 7.67)
July-September	2	14		1.27 (0.28, 5.71)
October-December	6	55		Referent

5.2.4 Discussion

The geographic and species distribution of ABLV

Evidence of ABLV infection in all four mainland species of flying fox, and in populations in eastern, northern and western Australia suggests that Australian bat lyssavirus is endemic in Australian flying foxes. The only surveyed state in which it was not detected was NSW, where the sample was limited to 31 *P. poliocephalus* (21 *Opportunistic* and 10 *Wild-caught*.). Thus by extension, the findings support the hypothesis that the existence of ABLV in Australian flying fox populations pre-dates its first description in 1996, and argue for its presence in Australia for at least the length of time necessary for it to establish in flying fox populations Australia-wide. As discussed in Chapter 3, the occurrence and distribution of flying foxes in Australia can be represented by a succession of overlapping foraging ranges centred on communal camps that are strategically scattered throughout the geographic range of each species. Groups of flying foxes move between camps depending of the availability of food resources near to each camp. Where geographic ranges of different species overlap, camps are commonly shared by species. Effective contact between populations is further facilitated by the periodic large-scale migration of *P. scapulatus* throughout their geographic range, which overlaps all others (Figure 5.1). This dynamic process, reflected in the genetic homogeneity of flying fox species across their Australian range (Sinclair *et al.*, 1996; Webb & Tidemann, 1996), is likely to promote the geographic and inter-species spread of ABLV, particularly as physical interaction between individuals of the same or different species occurs routinely in competition for

individual roosting sites, food resources, and mates. Molecular studies have provided further insight into the history of ABLV in Australia. While limited variation has been reported between isolates from flying foxes collected at different locations and at different times (Warrilow *et al.*, 2003), the isolation and subsequent sequencing of ABLV in the microchiropteran *Saccolaimus flaviventris* revealed substantial difference to the flying fox 'strain' (Gould *et al.*, 2002). This latter finding substantially strengthens the case for ABLV having existed in Australian bat populations for an extended period of time; long enough for strain differentiation within specific ecological niches to occur. The alternative explanation of multiple introductions of different ABLV variants to different taxonomic groups within the Australian bat population over time seems less plausible.

The prevalence of ABLV

The findings indicate that ABLV is a relatively rare infection in flying fox populations. Antigen was not detected in any of the wild-caught sample of 475 flying foxes (Table 5.3). Statistically, this is consistent with a prevalence of infection in the wild-caught study population of less than 1% (at a 95% confidence level, and assuming 100% test sensitivity). The 95% binomial confidence limits put the estimate somewhere between 0 and 0.7%. Given the probable <100% test sensitivity (see later discussion), the true value could be expected to be at the higher end of this interval, assuming test specificity is 100% While no comparative figures in megachiroptera could be found, Steece *et al* (1989) reported a similar prevalence of rabies infection (4/750, 0.5%) in wild-caught microchiroptera in New Mexico in the United States. In contrast to the findings in the wild-caught group, antigen was detected in 69/902 opportunistic flying foxes (Table 5.3), a crude prevalence of 7.6% (95% CI 6.0-9.6). This strong association between sampling methodology and FAT status warrants discussion. Intuitively, an infection that causes clinical disease[32] will be over-represented in the subset of the general population that is 'sick'. The opportunistic group in this study includes this population subset, consisting as it does of flying foxes debilitated by illness or injury. Logically therefore the prevalence of ABLV infection (that is, the proportion of FAT-positive individuals) should be higher in this group than in the wild-caught group. The public health implications stemming from this situation are discussed later.

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[32] Clinical histories associated with positive FAT status in flying foxes frequently include an inability to fly, hindquarter paresis, apparent general weakness, and behavioural changes including both aggression and depression (Field, HE: unpubl data).

The difficulty in detecting infections with a short clinical course, high case fatality rate and low prevalence using a cross-sectional study design are well recognized. Screening a positively biased sample is one approach to the problem, but it can limit understanding of the characteristics of the infection in the general population. An alternative is to increase the sample size for the general (wild-caught) population. This approach was untenable in this study, both from a resource and ethical standpoint[33]. Serology proved a useful alternative surveillance methodology. In contrast to the negative antigen (FAT) findings in the wild-caught sample discussed above, antibody was detected in eight of 266 wild-caught flying foxes (3%; 95%CI 1.3-5.8%) screened for antibody by RFFIT (Table 5.4). These individuals were clinically healthy and free of current infection based on the absence of antigen in brain smear by FAT. This presence of antibodies in the absence of current infection indicates non-fatal infection. Whether it indicates so-called 'aborted' infection, where an antibody response at the bite site eliminates the infection before virus enters the peripheral nervous system, or whether it indicates survival of clinical disease (see further discussion below) is unknown. Either interpretation indicates a mature host-parasite relationship, and in the case of lyssaviral infections (where case fatality rates typically approach 100%) strongly argues for co-evolution of the virus and host (McColl *et al.*, 2000). Antibody was also detected in 4/14 (28.6%; 95% CI 8.4-58%) opportunistically sampled flying foxes (Table 5.4). Two of these individuals were also positive by FAT, indicating current infection. Excluding these then, 2/12 (16.7%) opportunistically sampled flying foxes were seropositive in the absence of concurrent infection. This figure is nearly statistically significantly different (Fisher exact P=0.06) to the 8/266 (3%) seroprevalence in the wild-caught sample (Table 5.4). Other than chance, two plausible explanations fit this scenario: the RFFIT-positive, FAT-negative individuals were infected at the time but their infection status was misclassified because of a less than 100% sensitivity for the antigen-detecting FAT; or these were recovering (but debilitated) clinical cases which have survived at least long enough for antigen in the brain to be undetectable by FAT. Summers *et al* (1995) note that recovery after neurological disease has been reported with rabies, but that the phenomenon is much less common than aborted infection. Hooper *et al* (1997) also reported a seroprevalence of 16% (13/81) in flying foxes by RFFIT using rabies antigen. The sample was a non-random sample of 'healthy or sick' flying foxes submitted to AAHL for screening, and so probably overestimates non-fatal infections in

[33] The test to detect infection (antigen) is performed on brain tissue and therefore necessitates destructive sampling. To detect an infected wild-caught flying fox at say 0.1% prevalence it would be necessary to capture and kill 1000 flying foxes.

flying foxes[34]. Arguin *et al* (2002) reported a seroprevalence of 9.5% (22/231) in Philippine bats (mega- and microchiroptera) by RFFIT using specific ABLV antigen. All of these estimates contrast sharply with the 69% (514/750) rabies seroprevalence reported in a population of (microchiropteran) *Tadarida brasiliensis* in the United States (Steece & Altenbach, 1989). Apart from Hooper *et al* (1997), no comparative figures for lyssavirus seroprevalence in flying foxes exist.

Some discussion of the use of rabies virus in RFFIT screening for ABLV is timely. As noted earlier, ABLV and classical rabies virus both belong to lyssavirus serotype 1 (Fraser *et al.*, 1996) and consequently cross-neutralise. Thus, AAHL initially used a rabies virus RFFIT as the serologic test for ABLV. While the sensitivity and specificity of this test for ABLV were unknown, the initial validation of a later ABLV RFFIT developed at AAHL indicated good agreement between the two tests (Ross Lunt, AAHL; pers. comm.). Recently however, serologic investigations for lyssaviruses in bats in The Philippines has suggested the rabies RFFIT has a sensitivity of only 23% (5/22) relative to the ABLV RFFIT (22/22), at least on the ABLV-like virus in Philippine bats (Arguin *et al.*, 2002). Thus, notwithstanding the different criteria for positivity in the latter study[35], it is possible that serologic investigations in Australia using rabies virus RFITT have underestimated ABLV prevalence in Australian bats. It is also timely to note several important limitations to a serologic methodology in surveying for ABLV. Firstly the methodology precludes obtaining antigenic material to support molecular epidemiology studies; secondly, the current infection status of an individual cannot be determined; and thirdly, prevalence comparisons are only valid if the case fatality rate is equal in the compared groups.

Some discussion of the level of agreement between the standard FAT and the RFFIT is also warranted. Based on the Kappa statistic, agreement between the two is poor (Kappa = 0.18), however interpretation is meaningless given the substantial asymmetry along the concordant diagonal (Figure 5.2). The P (negative) and P (positive) calculations are more informative. The P (negative) of 0.97 indicates a high level of agreement in terms of negative results; that is, when one test is negative, there is a high probability that the

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[34] Both samples from which the seroprevalence estimate is derived include 'sick' flying foxes that would have died.

[35] In the Philippines study, a sample was defined as positive for neutralising antibodies if a 90% or greater reduction in infectious centers was seen relative to a positive control. The AAHL criterion for positivity of a 50% reduction is likely to have improved the sensitivity of the rabies-based RFFIT.

other will be. Conversely, the low P (positive) of 0.21 indicates poor agreement between positive tests. This is intuitively the case, given that one test measures antigen and the other antibody, and that in this study only 2/281 individuals screened for both antigen and antibody were positive for both.

Risk factors for infection with ABLV

Species and *Age* appear to be important risk factors for infection in flying foxes. Four variables were significantly associated with *FAT status* on univariate analysis – *Species*, *Age*, *SampleMethod* and *SampleLocation* (Table 5.3). Three were similarly associated with *RFFIT status* - *Species*, *SampleMethod* and *SampleLocation* (Table 5.4). The association with *SampleMethod* reflects the positively biased nature of a opportunistic sample where infection causes clinical disease; the association with *SampleLocation* reflects the association between *SampleMethod* and *SampleLocation*. When *Species* was stratified by *SampleMethod* to control for possible confounding, the association between *Species* and *FAT status* in the *Opportunistic* stratum was strengthened (Table 5.8). This is intuitively correct, because all the FAT-positives are in the opportunistic group, and the wild-caught individuals only increases the denominator. The zero positive values in the *Wild-caught* stratum precluded identification of any interaction. The association between *Species* (*P. scapulatus*) and *RFFIT status* (Table 5.4) further supports *Species* as a putative risk factor for infection. Here however, stratification by *SampleMethod* suggests interaction is occurring although the data is sparse (Table 5.12). Of the four species, *P. scapulatus* (Little red flying fox) is most strongly associated with a positive *FAT status* and a positive *RFFIT status*. While a positive FAT association could indicate either higher disease prevalence or a higher case fatality rate in *P. scapulatus*, the positive RFFIT association rules out the latter. The biology of *P. scapulatus* does differ in a number of major respects to the other mainland species, making plausible a hypothesis that host or environment factors might be responsible for an increased prevalence of infection in the species. For example, firstly, the roosting density (and therefore frequency of direct physical contact) of *P. scapulatus* is much greater than in other species (Hall & Richards, 2000) (Figure 5.3). Secondly, *P. scapulatus* undertake much larger nomadic movements than the other species, potentially increasing their opportunity for exposure(Hall & Richards, 2000). Thirdly, the reproductive cycle of *P. scapulatus* is countercyclical to the other species (Hall & Richards, 2000).

Figure 5.3: Roosting density of *P. scapulatus*

From Hall and Richards (2000)

Univariate analysis indicated that mature flying foxes in this study were more than twice as likely (RR=2.07) to be FAT-positive than were immature flying foxes (Table 5.3). Further, after stratifying by *SampleMethod* to control confounding, the strength of the association between *Age* and *FAT status* in the *Opportunistic* stratum was strengthened (Table 5.9). Age is commonly a risk factor for horizontally transmitted infectious diseases that provoke a persistent antibody response (Mills & Childs, 1998), older animals having had a greater (temporal) opportunity for exposure and infection. However, Steece *et al* (1989) found juvenile *T. brasiliensis* 3.5 times more likely to be antigen-positive (14/600, 2%) than mature individuals, contending that this indicated a peak exposure period shortly after birth. Notwithstanding, *T. brasiliensis* is a communal cave-dwelling microchiropteran species whose biology and ecology differs substantially from Australian flying foxes.

The near-significant univariate association between time of year and FAT status (p=0.067) (Table 5.15) disappeared when *P. scapulatus* was excluded from the analysis (p=0.29) (Table 5.16), indicating that *Species* was confounding the association. Nonetheless, the

possibility of a temporal association should not be completely discounted. After excluding *P. scapulatus*, flying foxes submitted in the second quarter remained 1.96 times more likely (95%CI 0.93 to 4.1) to be FAT-positive than those submitted in the referent last quarter (Table 5.16). Mating in these species typically peaks in March/April, so a higher relative risk in the second quarter could indicate an association between mating and the incidence of ABLV infection in these species. Certainly the increased number, and sometimes aggressive nature, of physical contact between individuals in the mating season make increased transmission of a bite-vectored infection plausible. However, in *P. scapulatus*, the statistically significant temporal association argues against an association with mating (Table 5.17). The reproductive cycle of *P. scapulatus* is countercyclical to the other species, with *births* peaking in March/April, thus a higher probability of infection in this species in the first quarter suggests an association between the pre-natal period and the incidence of ABLV infection. A biologically plausible rationale for such an association is elusive. As discussed above, Steece *et al* (1989) described an association between the post-natal period and the incidence of rabies infection in *T. brasiliensis*. Perez-Jorda *et al* (1995) reported seasonal variation in antibody titres to European bat lyssavirus 1 in a colony of *Eptesicus serotinus*, another microchiropteran species (with titres falling from a maximum of 74% in spring to less than 10% in summer), but failed to describe the variation in terms of the stages of the bats reproductive cycle.

Certainly the concept of temporal variation in exposure associated with seasonal behavior makes biological sense with flying foxes, and it is worthwhile considering another such behaviour – nomadic movement – in addition to reproduction. *P. scapulatus* is a highly mobile species, and large coalesced populations periodically make major nomadic movements in pursuit of preferred food trees (Hall & Richards, 2000). They use 'local' flying fox camps as daytime roosts, and typically swell camp numbers from thousands to tens or hundreds of thousands, with a resultant increase in density and physical interaction. Thus the higher probability of infection in all species in the first two quarters could reflect an association between the nomadic movements of *P. scapulatus* and the incidence of ABLV infection.

Opportunistic individuals were strongly associated with *FAT status* and *RFFIT status* on univariate analysis (Tables 5.3 & 5.4). Indeed, all antigen-positive bats were from the opportunistic subset. This finding is important from a public health perspective, as this subset presents the greatest opportunity for contact with people. These sick and injured

bats are typically collected and submitted for diagnosis by wildlife carers and members of the general public. McCall *et al* (2000), by analyzing demands for human post-exposure rabies vaccination, similarly identified persons and occupations having contact with sick and injured flying foxes as being more likely to suffer at-risk exposure.

The association between *SampleLocation* and *FAT status* (Table 5.3) and *SampleLocation* and *RFFIT status* (Table 5.4) is spurious. It directly reflects the association between *SampleMethod* and *FAT status* in that zero opportunistic samples came from *WA & NT* (Table 5.9), and *SampleMethod* and *RFFIT status* in that zero opportunistic samples came *WA & NT*, and zero wild-caught samples came from *Qld* (Table 5.14).

The widespread geographic and taxonomic occurrence of ABLV in flying foxes raises the question of the extent of infection in microchiropteran species. As discussed earlier, ABLV has been identified in *S. flaviventris*, and molecular studies confirm a separate and distinct 'clade' in this species. Also, the source of infection in the first human case of Australian bat lyssavirus has been ascribed to *S. flaviventris* (Hanna *et al.*, 2000), making determination of the extent of ABLV occurrence in microchiroptera important from public health perspective as well. Section 5.3 describes targeted surveillance of this group of bats.

5.3 SURVEILLANCE OF MICROCHIROPTERA FOR AUSTRALIAN BAT LYSSAVIRUS

5.3.1 Introduction

Australian microchiroptera exhibit a much great biodiversity than Australian megachiroptera, being represented by six families (Emballonuridae, Megadermatidae, Rhinolophidae, Hipposideridae, Vespertilionidae, and Molossidae) containing 20 genera and about 63 species (Hall & Richards, 2000). Typically, Australian microchiroptera (commonly known as microbats) are small, insectivorous and navigate using echo-location[36]. Representatives of three of the six families found in Australia have been associated with lyssavirus infections elsewhere in the world (McColl *et al.*, 2000), making it plausible that ABLV is prevalent in Australian microbats. Further, the ubiquitous nature of microbats in Australia, and the affinity of some species for urban areas (including

[36] The ghost bat *Macroderma gigas* (*Megadermatidae*) is a notable exception, being carnivorous and weighing 120 grams.

residential houses) underlines the importance of establishing the role of microbats in the ecology of Australian bat lyssavirus.

The primary objectives of this study were to

1. Describe the geographic and taxonomic distribution of ABLV infection in Australian microbats;
2. Estimate the prevalence of infection in surveyed microbat populations;
3. Identify animal-level risk factors for infection in microbats.

5.3.2 Materials and methods

5.3.2.1 Study design

The study took the form of a cross-sectional survey of non-randomly (purposively) selected microbat populations on mainland Australia. The study population comprised known accessible roosts in each surveyed state, and microbats coming into human care in Queensland and New South Wales. The individual was the unit of interest. Samples taken from individuals consisted of either brain only, brain and blood, or blood only.

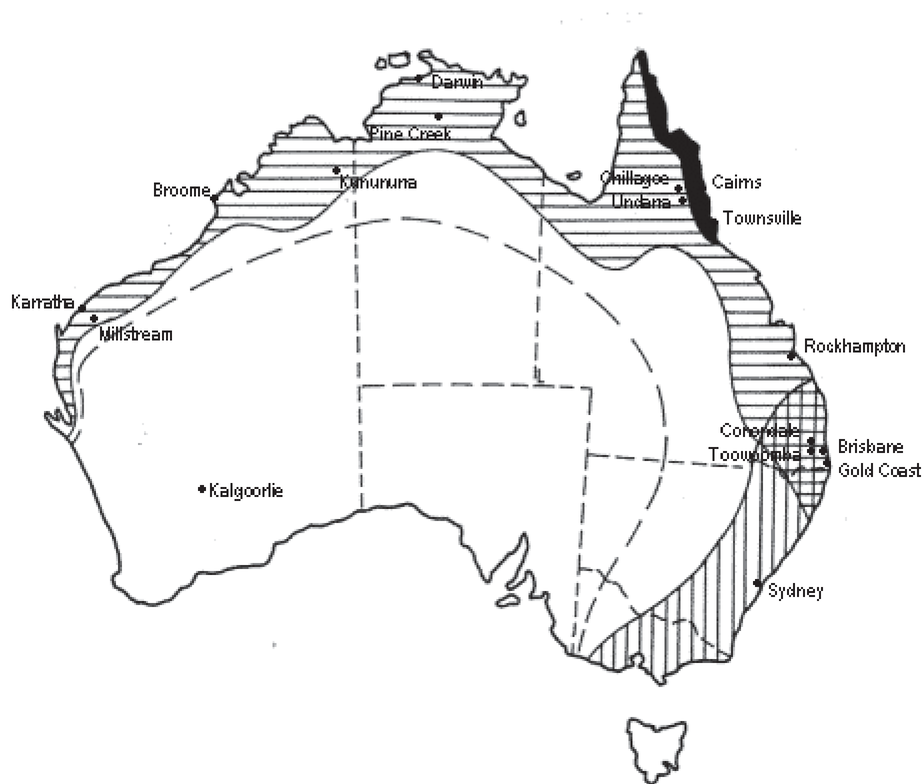
5.3.2.2 Sampling methodology

Bats were sampled by one of two methodologies - wild-caught or opportunistic. Wild-caught bats were collected by mist-net (Queensland, Northern Territory) and by shooting (Western Australia). The methodologies are described in detail in Chapter 3 (3.3.2.3). Opportunistic bats consisted of sick, injured or recently dead individuals submitted to YVL by wildlife authorities, wildlife rescue groups, or members of the general public.

5.3.2.3 Sampling locations

Wild-caught bats were collected from multiple locations in Queensland, New South Wales, the Northern Territory, and Western Australia. Opportunistic samples were primarily obtained from wildlife authorities, carers, and institutions in the greater Sydney area in New South Wales, and in the greater Brisbane area, the Darling Downs, the Gold Coast, and Townsville in Queensland. See Figure 5.4.

Figure 5.4: Primary sampling locations of microchiropteran bats and the distribution of flying foxes on mainland Australia[1].



Key: Horizontal hatching = *P. alecto*
Vertical hatching = *P. poliocephalus*
Solid black = *P. conspicillatus*
Broken line = southern inland limit of *P. scapulatus*.

[1] Adapted from Hall and Richards (2000).

5.3.2.4 Sample size

As described in Section 5.2.2.4.

5.3.2.5 Sampling period

The sampling period in Queensland was April 1996 to March 2001; in New South Wales, November 1996 to October 1998; in the Northern Territory, August 1999 and March 2001; and in Western Australia, December 1998, February 2001 and June 2001.

5.3.2.6 Specimen collection

As described in Section 5.2.2.6. Where conservation biodiversity imperatives precluded bats being killed, blood was taken by jugular bleed and the bats were released.

5.3.2.7 Laboratory methodologies

As described in 5.2.2.7.

5.3.2.8 Data management and statistical analysis

Data were recorded on the outcomes variables FAT status and RFFIT status, and on six possible explanatory variables: genus[37], sex, age, sampling location, sampling year, and sampling method. Age class (immature, mature) was determined where possible, based on morphometrics including bodyweight and length. All data were stored and managed in a Microsoft ™ Access 97 ™ database. Data was exported to Microsoft ™ Excel 97 ™ for descriptive and univariate analyses. EpiInfo (Version 6) Statcalc was used in the latter to test for associations between each of the outcome variables and the possible explanatory variables, with Chi square *p* values indicating the statistical significance of associations, and unadjusted relative risk (RR) indicating the magnitude of associations. Yates Corrected *p* value was used except where cell values were less than 5, when Fisher's Exact *p* value was used. Stratified analysis was used to detect confounding and/or interaction between the possible explanatory variables.

5.3.3 Results

A total of 1234 bats from at least 14 genera of microbats found on mainland Australia, and from locations in Queensland, New South Wales, the Northern Territory and Western Australia, were surveyed over a five-year period. Evidence of current or previous ABLV infection was found in 7/14 known genera surveyed (Table 5.18, 5.19 & 5.20) and in three of the four states surveyed. Samples taken from each individual were either brain (844), brain and blood (318), or blood (72). The *brain only* group included opportunistic and wild-caught bats; the *brain and blood* group and the *blood only* group contained only wild-caught bats (Figure 5.5). Of the *brain only* group, 5/844 (0.59%) were positive for antigen (Table 5.18). All five were opportunistic bats, giving a prevalence of 5/176 (2.8%) in the opportunistic subset of this group. Of the *brain and blood* group, none (0/318) were positive for antigen, and 9/318 (2.8%) were positive for antibody (Table 5.19). Of the latter, 3 were from locations outside the known range of flying foxes (Figure 5.4). Of the *blood only* group, 1/72 (1.38%) was positive for antibody (Table 5.20). Of the total 15 bats

[37] Some bats could not be accurately classified to Species level.

positive by either test (Table 5.21), 8 (53%) were *S. flaviventris*, with 5/8 positive by FAT and 3/8 positive by RFFIT (Table 5.21). No bats were positive by both tests.

Figure 5.5: Composition of the non-random sample of 1234 microbats from Queensland, New South Wales, the Northern Territory and Western Australia collected between April 1996 and June 2001 sample ram of the sample composition.

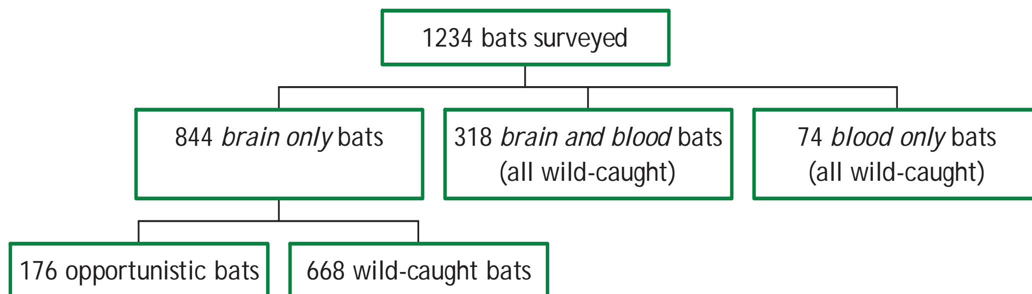


Table 5.18: Characteristics of 844 *brain only* group microbats non-randomly sampled from Queensland, New South Wales, the Northern Territory and Western Australia between April 1996 and June 2001 that were screened for ABLV by FAT.

	Number of bats tested (positive) by FAT		
	Total	Opportunistic	Wild-caught
Genus			
Chaerophon	2		2
Chalinolobus	28	22	6
Miniopterus	362	29	333
Mormopterus	252	19	233
Myotis	22	2	20
Nyctophilus	25	24	1
Saccolaimus	10 (5)	8(5)	2
Scotorepens	84	22	62
Taphozous	5	2	3
Vespedalus	10	4	6
Unrecorded	44	44	
Sex			
Male	253 (1)	70(1)	183
Female	462 (2)	49(2)	413
Unknown	129 (2)	57(2)	72
Age			
Immature	9	8	1
Mature	346 (3)	49(3)	413
Unknown	489 (2)	86(2)	403
SampleMethod			
Wild-caught	668		668
Opportunistic	176 (5)	176(5)	
SampleLocation			
NT	20		20
Qld	776 (5)	155(5)	621
WA	21		21
NSW	27	21	6
SampleYear			
1996	12 (2)	12(2)	
1997	702 (2)	82(2)	620
1998	43 (1)	27(1)	16
1999	41	40	1
2000	16	10	6
2001	30	5	25
Total	844 (5)	176	668

Table 5.19: Characteristics of 318 *brain and blood* group microbats non-randomly sampled from the Northern Territory and Western Australia between December 1998 and June 2001 that were screened for ABLV by both FAT and RFFIT.

Variable	Number of bats tested		
	Total	Positive by	
		FAT	RFFIT
Genus			
Chaerophon	2		1
Chalinolobus	55		2
Hipposideros	30		1
Miniopterus	60		
Mormopterus	3		
Myotis	14		
Nyctophilus	1		
Saccolaimus	24		3
Scotorepens	2		
Tadarida	45		1
Taphozous	37		
Vespedalus	45		1
Sex			
Male	132		1
Female	185		8
Unknown	1		
Age			
Immature	5		
Mature	202		3
Unknown	111		6
SampleMethod			
Wild-caught	318		9
Opportunistic			
SampleLocation			
NT	114		
WA	204		9
SampleYear			
1998	46		4
1999	64		
2000			
2001	208		5
Total	318		9

Table 5.20: Characteristics of 72 *blood only* group microbats non-randomly sampled from the Northern Territory and Western Australia between December 1998 and May 2001 that were screened for ABLV by RFFIT.

Variable	Number of bats tested (positive) by RFFIT
Genus	
Macroderma	68 (1)
Taphozous	4
Sex	
Male	34
Female	30
Unknown	8 (1)
Age	
Immature	
Mature	72 (1)
SampleMethod	
Wild-caught	72 (1)
Opportunistic	
SampleLocation	
NT	46 (1)
WA	26
SampleYear	
1998	4
1999	
2000	
2001	68 (1)
Total	72 (1)

Table 5.21: Characteristics of 15 microbats non-randomly sampled from Queensland, the Northern Territory and Western Australia between April 1996 and June 2001 that tested positive for ABLV either by FAT or RFFIT.

Variable	Number of bats tested		
	Total	Positive by	
		FAT	RFFIT
Genus (Family)			
Chaerophon (*Molossidae*)	1		1
Chalinolobus (*Vespertilionidae*)	2		2
Hipposideros (*Hipposideridae*)	1		1
Macroderma (*Megadermatidae*)	1		1
Saccolaimus (*Emballonuridae*)	8	5	3
Tadarida (*Molossidae*)	1		1
Vespedalus (*Vespertilionidae*)	1		1
Sex			
Male	2	1	1
Female	10	2	8
Unknown	3	2	1
Age			
Immature			
Mature	7	3	4
Unknown	8	2	6
SampleMethod			
Wild-caught	10		10
Opportunistic	5	5	0
SampleLocation			
NT	1		1
Qld	5	5	
WA	9		9
SampleYear			
1996	2	2	
1997	2	2	
1998	5	1	4
1999			
2000			
2001	6		6
Total	15	5	10

Univariate analysis of the association between the possible explanatory variables and *FAT status* (that is, antigen status) was carried out on the *Opportunistic* subset of the *brain only* group (Table 5.22). The analysis identified *Genus* (p<0.001) as significantly associated with the outcome variable.

Univariate analysis of the association between the possible explanatory variables and *RFFIT status* (that is, antibody status) was carried out on a reduced dataset of genera where one or more individuals were RFFIT-positive (Table 5.23). The analysis identified *Genus* (p=0.005) and *SampleYear* (p=0.01) as significantly associated with the outcome variable. Zero immature age class values precluded the inclusion of *Age* in the analysis. An additional univariate analysis showed the association between *SampleYear* and *RFFIT status* was confounded by *Genus* (Table 5.24).

Table 5.22: Univariate association between a number of independent variables and FAT status in 176[1] microbats non-randomly sampled from the Queensland and New South Wales between April 1996 and June 2001.

Variable	Number of bats testing		P value	Relative Risk (95%CI)
	FAT positive	FAT negative		
Genus			<0.001	
Saccolaimus	5	3		
Other genera	0	168		Unable to be calculated[4]
Sex[2]			0.57	
Male	1	69		Referent
Female	2	47		2.86 (0.27, 30.64)
Age[3]			1.00	
Mature	3	79		
Immature[4]	0	8		Unable to be calculated[5]
SampleMethod			Unable to be calculated[4]	
Wild-caught	0	0		
Opportunistic	5	171		Unable to be calculated[5]
SampleLocation			1.00	
Qld	5	150		
NSW	0	21		Unable to be calculated[5]
SampleYear[6]			0.33	
1996-98	5	116		
1999-01	0	55		Unable to be calculated[5]
Total	5	171		

[1] The *Opportunistic* subset from Table 5.18. No FAT-positives were found in the *Wild-caught* subset so it was excluded from this analysis.
[2] 57 individuals of unknown Sex (including 2 FAT positives) were excluded from the analysis.
[3] 86 individuals of unknown Age (including 2 FAT positives) were excluded from the analysis.
[4] The zero value precludes calculation, but for illustration, if 0 is replaced by 1, the relative risk is 105.6 (13.92, 801.48).
[5] The zero values preclude calculation.
[6] Years 1996-1998 and 1999-2001 data combined

Table 5.23: Univariate association between a number of independent variables and RFFIT status in 269[1] *brain and blood* group and *blood only* group microbats non-randomly sampled from the Northern Territory and Western Australia between December 1998 and June 2001 whose genera were RFFIT-positive.

Variable	Number of bats testing		P value	Relative Risk (95%CI)
	RFFIT positive	RFFIT negative		
Genus[2]			0.005	
Macroderma	1	67		Referent
Tadarida	1	44		1.51 (0.1, 23.55)
Vespedalus	1	44		1.51 (0.1, 23.55)
Hipposiderus	1	29		2.27 (0.15, 35.05)
Chalinolobus	2	53		2.47 (0.23, 26.56)
Saccolaimus	3	21		8.5 (0.93, 77.86)
Chaerophon	1	1		34 (3.12, 370.58)
Sex[2]			0.15	
Male	1	100		Referent
Female	8	151		5.08 (0.65, 40.23)
Age[3]			Unable to be calculated[4]	
Mature	4	159		
Immature	0	0		Unable to be calculated[4]
SampleMethod			Unable to be calculated[4]	
Wild-caught	10	259		
Opportunistic	0	0		Unable to be calculated[4]
SampleLocation			0.17	
NT	1	86		Referent
WA	9	173		4.3 (0.55, 33.42)
SampleYear[5]			0.01	
1998-99	4	25		Referent
2000-01	6	234		0.18 (0.05, 0.60)
Total	10	259		

[1] From Table 5.19 and 5.20. All bats in the *brain and blood* group (Table 5.19) and the *blood only* group (Table 5.20) were wild-caught.
[2] Nine individuals of unknown Sex (including 1 RFFIT positive) were excluded from the analysis.
[3] 106 individuals of unknown Age (including 6 RFFIT positives) were excluded from the analysis.
[4] The zero values precludes calculation.
[5] Years 1998-1999 and 2000-2001 data combined

Table 5.24: Univariate association between *Species* and *SampleYear* in 269[1] *brain and blood* group and *blood only* group microbats non-randomly sampled from the Northern Territory and Western Australia between December 1998 and June 2001 whose genera were RFFIT-positive.

	SampleYear		P value
	98-99	00-01	
Genus			>0.001
Macroderma		68	
Tadarida		45	
Vespedalus		45	
Hipposiderus		30	
Chalinolobus	3	52	
Saccolaimus	24		
Chaerophon	2		

[1] Table 5.23 data

5.3.4 Discussion

The geographic and species distribution of ABLV

Evidence of either current or previous infection with ABLV was found in 7/14 known genera surveyed, and thus in 5/6 families of microbats found in Australia. Further, bats testing positive by either FAT or RFFIT came from populations in eastern, northern and western Australia. These findings suggest that Australian bat lyssavirus is endemic in Australian microbats. The only surveyed state in which it was not detected was NSW, where the sample was limited to 27 *brain only* bats (21 opportunistic and 6 wild-caught bats). These findings further support the hypothesis (posed in Section 5.2.4) that the existence of ABLV in Australian bat populations pre-dates its first description in 1996, and argue for its presence in Australia long enough for it to establish in mega- *and* microchiropteran populations Australia-wide. Importantly, the finding of antibody-positive individuals in non-nomadic populations beyond the known range of flying fox species strongly suggests that ABLV is cycling independently in microbat populations. Molecular studies to date support this contention, having identified consistent sequence differences between isolates from microchiropteran and megachiropteran species (Gould *et al.*, 2002).

Some discussion of the absence of evidence of infection in Australian *Miniopterus* species

is warranted. *M. schreibersi* and *M. australis* are ubiquitous in Australia, and large mixed colonies frequently occur in urban areas. As lyssavirus infections had been previously detected in *Miniopterus* elsewhere in the world (McColl *et al.*, 2000), the genus was seen as a key ABLV surveillance target. It attracted the greatest sampling intensity, with a total of 422 collected from multiple disparate locations in Queensland and one location in NSW. The absence of detectable antigen in any of 362 *brain only* bats (29/362 were opportunistic bats) (Table 5.18), or antibody in any of the 60 *brain and blood* bats (Table 5.19) suggests either a very low prevalence of infection, a high case fatality rate, or an absence of infection in the *Miniopterus* study population. These findings contrast sharply with those of a recent survey of Philippine bats where neutralising antibodies to ABLV were most prevalent in *M. schreibersi* (4/11, 36%) (Arguin *et al.*, 2002). One plausible explanation is that ABLV infection may be episodic (particularly in smaller bat colonies) and neutralising antibody short-lived. McColl *et al* (McColl *et al.*, 2000) report short-lived titres to ABLV and rabies following peripheral experimental inoculation, but little information is available on antibody persistence following natural infection. While a number of authors report high lyssavirus seroprevalence in wild microbat populations[38], it is unclear whether these findings indicate antibody persistence or repeated exposure. The seasonal variation in anti-EBL1 antibody titre described by Perez-Jorda *et al* (1995) supports the latter.

The absence of evidence of infection in other surveyed genera also warrants discussion. Limited sample size precludes meaningful interpretation for some genera (Tables 5.18, 5.19 & 5.20), while for others the possible interpretations discussed above may be valid (that is, low prevalence of infection, high case fatality rate, episodic infection, short-lived antibody titres). Notwithstanding, the biological and ecological diversity within the sub-order *Microchiroptera* is marked - some are gregarious, others solitary, some are seasonally nomadic, others are sedentary - and these factors are likely to underscore the incidence and maintenance of infection in a particular genera or species.

The prevalence of ABLV

ABLV infection appears to be rare in Australian microbats. Infection (antigen) was not detected in any wild-caught bat, based on FAT screening of 668 *brain only* bats (Table

[38] Perez-Jorda *et al* (1995) reported a 74% seroprevalence to European bat lyssavirus 1 in *Eptesicus serotinus*; Steece *et al* (1989) reported 69% seroprevalence to rabies in *Tadarida brasiliensis* in the United States.

5.18) and 318 *brain and blood* bats (Table 5.19). Statistically, this gives a point prevalence estimate of 0% in the wild-caught study population[39]. The 95% binomial confidence interval (CI) is 0 to 0.37%. This is consistent with findings of bat lyssavirus infections elsewhere. Steece *et al* (1989) report a comparable rabies infection prevalence (4/750, 0.5%) in wild-caught microbats in New Mexico in the United States, albeit that their sample was from a large single-species colony. Arguin *et al* (2002) comment that most studies of healthy bats have found a low prevalence of active infection, usually <1%. In contrast to the wild-caught subset, antigen was detected in 5/176 opportunistic bats (Table 5.18), a crude prevalence of 2.8% (95%CI 0.9-6.5). The strong association between sample methodology and FAT status seen with flying foxes was, as expected, evident in microbats as well. As previously argued (Section 5.2.4), because ABLV causes clinical disease, and because the opportunistic subset includes clinical cases, the prevalence of ABLV infection (as measured by the proportion of FAT-positive individuals) can be expected to be higher in the opportunistic subset than in the wild-caught subset.

Serology again proved a useful alternative surveillance methodology. In contrast to the negative antigen (FAT) findings in the wild-caught subset discussed above, antibody was detected in 9/318 bats in the *brain and blood* group (Table 5.19) and 1/72 bats in the *blood only* group (Table 5.20), giving a crude antibody prevalence of 2.6% (95% binomial CI 1.2-4.7). The former were clinically healthy and free of current infection based on the absence of antigen in brain smear by FAT. As previously discussed (Section 5.2.4), this scenario suggests non-fatal infection, and argues for a mature host-parasite relationship. Seroprevalence to bat lyssavirus infections reported elsewhere varies - 9.5% (Arguin *et al.*, 2002), 40.5% (Price & Everard, 1977), 69% (Steece & Altenbach, 1989) - but differences in methodology and sample composition preclude direct comparisons. Arguin *et al* (2002) assumed an antibody/antigen ratio of 100:1 in healthy bat populations.

Particular discussion of the association between the genus *Saccolaimus* and *FAT status* and *RFFIT status* is warranted. Of the total 15 bats positive by either test, 8 (53%) were *Saccolaimus*[40], including 5/5 (100%) of the FAT-positive bats and 3/10 (33.3%) of the RFFIT-positive bats detected in the study. Of the 8 opportunistic *S. flaviventris* screened by FAT, 5 (62.5%) were positive, and of the 24 *S. flaviventris* screened by RFFIT, 3 (12.5%)

[39] At a 95% confidence level, and assuming 100% test sensitivity.

[40] All were *Saccolaimus flaviventris*.

were positive. These findings suggest an important role for *S. flaviventris* in the ecology of ABLV. This is discussed further below.

Risk factors for infection with ABLV

Species appears to be the only risk factor for infection in microchiroptera, being the only variable significantly associated with *FAT status* on univariate analysis (Table 5.23). Two variables were significantly associated with *RFFIT status* on univariate analysis – *Species* and *SampleYear* (Table 5.23). The latter apparent association was due to confounding by *Species* (Table 5.24). *S. flaviventris* is statistically associated with a both positive *FAT status* (p<0.001; Table 5.22) and a positive *RFFIT status* (p=0.053; Table 5.23). If correct, these findings suggest *S. flaviventris* may be important in the maintenance of ABLV in microbats. Excluding chance, alternate explanations for this association are that the sample was biased towards *S. flaviventris*, or that test validity differed for *S. flaviventris*. Both scenarios seem implausible. There is no apparent reason why antigen-positive *S. flaviventris* would be more likely to be submitted for diagnosis than antigen-positive bats of other species, nor why antibody-positive *S. flaviventris* would be more likely to be wild-caught than antibody-positive bats of other species. Likewise, there is no plausible reason why the sensitivity of both tests should be increased or specificity of both decreased for *S. flaviventris*. One plausible explanation for the apparent increased infection prevalence is that the clinical course is longer in *S. flaviventris.* Further, the apparent increased antibody prevalence could follow a lower case fatality rate and/or longer antibody persistence. Both suggest a special relationship with the virus. The limited molecular studies are consistent with such a scenario, at least (as discussed earlier) to the extent that sequencing has demonstrated an ABLV variant in *S. flaviventris* distinct from that in flying foxes (Gould *et al.*, 2002). The absence of isolates or antigenic material from other microchiropteran species precludes further comparison.

All antigen-positive bats were again from the opportunistic subset (Table 5.18 & 5.19). As with flying foxes (Section 5.2.4), it is this group that presents the greatest opportunity for contact with people. These sick and injured bats are typically collected and submitted for diagnosis by wildlife carers and members of the general public, and thus represent a major risk for human exposure. Yet this long-standing risk behavior prompts a fundamental question of why ABLV appears to have only recently emerged, when mounting evidence suggests its long presence in Australian bat populations. Section 5.4 presents a retrospective investigation of archived bat specimens.

5.4 RETROSPECTIVE SURVEILLANCE OF BATS FOR EVIDENCE OF ABLV INFECTION

5.4.1 Introduction

An understanding of the temporal history of Australian bat lyssavirus in Australian bats is relevant to an understanding of the ecology of the virus and to the development of appropriate risk management procedures. ABLV was first described in May 1996 in flying foxes (Fraser *et al.*, 1996) and shortly after (October 1996) in humans (Allworth *et al.*, 1996), yet the findings in the previous sections indicate that infection is endemic in Australian bats. Prophetically, in 1989 St. George (St. George, 1989), suggested the possibility of an undiscovered rabies-like virus in Australian bats when postulating the origins of Adelaide River virus (an ephemerovirus with a weak antigenic relationship to rabies virus). St. George went further, suggesting that the typically low prevalence of the rabies-related viruses in bats meant that an Australian bat lyssavirus might not become evident unless active surveillance of bats was undertaken, or unless a human or a domestic animal case was identified.

This study aimed to identify evidence of historic ABLV infections in Australian bats.

5.4.2 Materials and methods

5.4.2.1 Study design

The study took the form of a cross-sectional survey of archived materials. The study population(s) comprised the archived specimens available: formalin-fixed bats and frozen serum samples. The individual was the unit of interest.

5.4.2.2 Sampling location

Archived specimens were obtained from the Queensland Museum (formalin-fixed flying foxes and microbats), the University of Queensland (formalin-fixed microbats, and flying fox sera) and the CSIRO Long Pocket Laboratory in Brisbane (flying fox sera). The specimens were originally collected from multiple Queensland locations.

5.4.2.3 Sampling methodology

The formalin-fixed bat sample included all the specimens available from the University of Queensland and a non-random purposive sample of specimens from the Queensland

Museum. The QM collection included specimens acquired by shooting or mist-netting (that is, wild-caught) as well as specimens found dead, sick or injured. Effort was made to positively bias the study sample by over-representing the latter group and the species *Pteropus scapulatus* and *Saccolaimus flaviventris*[41]. The serum sample comprised all available specimens.

5.4.2.4 Sample size

Constraints on the availability of samples precluded the target minimum sample size of 60 individuals per species sought in the other studies. The maximum number available for each species was screened.

5.4.2.5 Sampling period

Effort was made to include specimens over several decades. The QM specimens were collected between 1946 and 1981. The UQ bat specimens were collected in 1975 and in 1993, and the sera between 1982 and 1984. The CSIRO sera were collected in 1976.

5.4.2.6 Specimen collection

Brain tissue was collected from the bats using a minimal impact technique. Typically, the skin, temporal muscle, cranium and dura were reflected forward to allow the discrete removal of one cerebral hemisphere. The specimens were subsequently returned to their source institutions. Sera were aliquoted from archived samples of sufficient volume. All work was performed in a class 2 biosafety cabinet in a PC3 laboratory.

5.4.2.7 Laboratory methodologies

Brain samples were tested by immunohistochemistry (IHC) using a biotin-streptavidin peroxidase-linked detection system at AAHL. Some samples were screened by histopathological examination at either YVL or AAHL prior to IHC. These (formalin-fixed) samples were paraffin-embedded, and stained with haematoxylin and eosin prior to examination. Samples regarded as suspect by either test were subject to real-time PCR assay (Taqman™) at Queensland Health Scientific Services Laboratory, Brisbane. Serology was performed at AAHL using a RFFIT incorporating either rabies virus antigen or (later) rabies virus antigen and specific ABLV antigen in parallel.

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[41] These species were shown to be positively associated with ABLV infection (measured by FAT status) in Sections 5.2 and 5.3).

5.4.2.8 Data management and statistical analysis

Data were recorded on the outcomes variables IHC status, RFFIT status and PCR status, and on six possible explanatory variables: species, age, sex, location of collection, year of collection, and method of collection. All data were stored and managed in a Microsoft ™ Access 97 ™ database. Data was exported to Microsoft ™ Excel 97 ™ for descriptive analysis.

5.4.3 Results

A total of 137 microbats from 10 species, and from multiple locations in Queensland were tested for evidence of ABLV infection by IHC (66) (Table 5.25) or RFFIT (71) (Table 5.26). Inadequate formalin fixation precluded interpretation by IHC in 12/66 brains. The remaining 54 were negative by IHC. Of the total 66 brains tested by IHC, 56 were screened by histological examination prior to IHC. Inadequate formalin fixation precluded interpretation in 5/56. Of the remaining 51, 49 were unremarkable, but 2 (both *S. flaviventris*) showed round basophilic structures consistent with Negri bodies[42] on histological examination. One of these also had a perivascular cuff of mononuclear cells sufficient to suggest mild encephalitis. These two brains and those of two other *S. flaviventris* that had clinical histories consistent with ABLV[43] were tested by PCR. All four were negative by Taqman™ PCR. Of the 71 serum samples, none had detectable antibodies to ABLV.

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[42] Negri bodies are single or multiple ovoid intracytoplasmic inclusions found in the CNS and previously crucially associated with the diagnosis of lyssaviruses Summers *et al* (1995).

[43] Both these additional bats were negative by IHC and unremarkable by histological examination.

Table 5.25: Characteristics of 66 non-randomly sampled bats retrospectively screened for Australian bat lyssavirus by histopathological examination, IHC and PCR.

Species	Number of bats tested			
	Total	by		
		Histology	IHC	Taqman™ PCR
Saccolaimus flaviventris	28	28	28	4
Pteropus scapulatus	23	12	13	
Pteropus alecto	25	4	5	
Miniopterus spp.	14	12	14	
Rhinolophus sp.	2		2	
Hipposideros sp.	1		1	
Kerivoula papuensis	1		1	
Scotorepens sp.	1		1	
Syconycterus sp.	1		1	
Total	66	56	66	4

Table 5.26: Characteristics of 71 non-randomly sampled flying foxes retrospectively screened for Australian bat lyssavirus by RFFIT.

Species	Number of bats tested (positive)
Pteropus poliocephalus	41
Pteropus scapulatus	10
Pteropus alecto	20
Total	71

5.4.4 Discussion

The lack of confirmed evidence of infection in the archived samples does not allow confident rejection of the hypothesis that ABLV is not a recent introduction to the Australian zoogeographic region. The 95% binomial CI on the 0/54 result in the antigen (brain) study is 0 to 6.6%, and on the 0/71 result in the antibody (serum) study, 0 to 5% (assuming 100% test sensitivity and specificity). The power of both studies has been

seriously limited by sample size and an inability to effectively increase the pre-test probability of infection. In the antigen study, the effective sample size was reduced to 54 by inadequate formalin fixation, and while the sample included 28 *S. flaviventris*, all were wild-caught. Only 4 of the total 66 bats in the antigen study were sick or injured. Based on data from Section 5.2 and 5.3, the sample would have needed to include 13 sick or injured flying foxes, or 35 sick or injured microbats for one to be antigen-positive[44]. In the antibody study, only 10/71 were *P. scapulatus*. Based on Section 5.2, the sample would have needed to include 15 *P. scapulatus* for one to be antibody-positive[45]. In both studies, the pre-test probability of infection achieved was well short of the approximately 5% dictated by a sample sizes of 66 (antigen study) and 71 (antibody study) as being necessary to enable determination of the presence/absence of infection with 95% confidence, and then assuming test sensitivity and specificity of 100%.

Unknown test sensitivity was likely an additional factor limiting the power of the antigen study. The sample consisted of formalin-fixed tissues, which precluded the use of the gold-standard FAT (used in Sections 5.2 & 5.3). The alternatives were histological examination, IHC and PCR. Histopathological examination was known to lack sensitivity. Hooper *et al* (1999) found eight of twenty-one (38%) confirmed ABLV-infected bats[46] to have no inflammation or only sparse inflammation by histopathological examination. The authors further noted that IHC staining in the 21 confirmed positive bats varied from widespread to localized, and recommended that a wide range of brain tissue from each animal be examined to maximize the sensitivity of both methods. The long-term storage of the archived bats in alcohol may also have affected the sensitivity of the IHC and PCR tests. While no literature could be found, it is probable that the sensitivity of both tests would be reduced, though to an unknown degree. Thus it was recognized at the study design stage that the sensitivity of all available tests was limited and that, while parallel test interpretation would improve sensitivity, negative study findings would be inconclusive.

[44] In Section 5.2, 0/475 wild-caught flying foxes but 69/902 opportunistic flying foxes were antigen-positive (Table 5.3); in Section 5.3, 0/668 wild-caught microbats but 5/176 opportunistic microbats were antigen-positive (Table 5.15).

[45] In Section 5.2, 8/117 wild-caught *P. scapulatus* were antibody-positive (Table 5.12).

[46] The 21 bats (19 flying foxes and 2 *S. flaviventris*) were confirmed as ABL infected by FAT, virus isolation, PCR and/or IHC.

The findings of the histopathological examination on the two *S. flaviventris* warrant discussion. Both bats had basophilic structures consistent with Negri bodies on histopathological examination. One bat also had a perivascular cuff of mononuclear cells sufficient to suggest mild encephalitis. While lymphocytic perivascular cuffing is not specific to lyssaviral infections, the presence of Negri bodies has been considered so[47]. However there is now some doubt about the specificity of the presence of Negri bodies, following the description of Negri body-like inclusions[48] in normal animals and in other neurological diseases (Summers *et al.*, 1995). Notwithstanding, both these bats were *S. flaviventris*, a species known to be strongly associated with ABLV infection (see Section 5.3), and both were collected from the same property within 2 days of each other. If the presence of Negri bodies in these two bats was associated with ABLV infection then they indicate that ABLV was cycling in a north Queensland bat population in 1981, 15 years before it was first described, and 14 years before the earliest previously reported case in bats[49].

5.5 CHAPTER CONCLUSIONS

Australian bat lyssavirus has a wide geographic and taxonomic distribution in Australian flying foxes and microbats, and can be regarded as endemic in Australian bat populations. The serologic findings in clinically normal bats indicate that non-fatal infection is not infrequent and further supports the contention of endemicity. While infection is relatively rare in wild-caught populations, it is over-represented in the population subset of sick and injured bats rescued by people, and thus poses a significant public health risk.

Serology is a valuable tool for ABLV surveillance in flying foxes and microbats in that it provides a more efficient and generally non-destructive mechanism for detecting infection at a population level. However small blood volumes and limited serum harvests from many microbat species present challenges. In the Australian context, it is likely that RFFIT validity can be maximized by using an ABLV antigen rather than rabies antigen.

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[47] While the presence of Negri bodies may be specific for lyssaviral infections, sensitivity is poor. Hooper *et al* (1999) observed Negri bodies in only 9/21 (43%) confirmed ABL-infected bats.

[48] So called pseudo-Negri bodies.

[49] Speare *et al* (1997) retrospectively identified ABL in a *P. alecto* in January 1995, 16 months prior to its first description in May 1996 (see Chapter 7).

Species appears to be an important animal-level risk factor for ABLV infection in flying foxes and microbats. *P. scapulatus* exhibited a higher antigen and antibody prevalence than other flying fox species. *S. flaviventris* was the only microbat species in which antigen was detected, and it had a significantly higher antibody prevalence than other species. These findings suggest an important role for these species in the ecology of ABLV. Further work is needed to understand the mechanism of maintenance of infection in flying fox and microbat populations. Molecular epidemiology studies targeting microbats are also a research priority.

The retrospective study produced no conclusive evidence of historic ABLV infection in bats but the limited power of the study does not allow confident rejection of the hypothesis that ABLV is not a recent introduction to the Australian zoogeographic region.

The fundamental question of why ABLV has only recently 'emerged' in humans, when mounting evidence indicates not only its long presence in Australian bat populations but also likely opportunity for human exposure associated with flying fox rescue and rehabilitation, remains unanswered. Could changed seasonal movement patterns by *P. scapulatus* have resulted in increased exposure and infection in urban flying fox populations, and thus humans? This hypothesis will be presented and discussed in Chapter 7.

CHAPTER SIX

A DETERMINISTIC MODEL OF HENDRA VIRUS INFECTION DYNAMICS IN A FLYING FOX POPULATION

6.1 Introduction

The mode of transmission of Hendra virus in flying foxes is unknown, as are the routes of excretion and infection. Chapter 3 suggests that horizontal transmission is the primary mode of transmission in free-living populations, while the findings of the study of the captive colony (Chapter 4) were more consistent with vertical transmission. Consequently, key questions remain unresolved, including:

- What factors govern the dynamics of infection in flying fox populations?
- What factors are important in the maintenance of infection in flying foxes?

The objective of this chapter was to develop a series of deterministic state-transition models (Graat & Frankena, 2001) to examine the dynamics of HeV infection in a hypothetical flying fox population. Of specific interest were

1. the key parameters determining the rate of spread of infection in a naive population (Model 1).
2. the key parameters determining the maintenance of infection in an infected population (Model 2).

6.2 Materials and methods

6.2.1 Model 1

A simple deterministic state transition model was developed for a hypothetical population of flying foxes. The following assumptions were made:

- The size of the population was constant, with births, deaths, immigration and emigration assumed to be zero.
- Infection was directly transmitted.
- All flying foxes were equal with respect to susceptibility and infectivity.
- Following infection, each flying fox was uniformly infectious throughout its infectious period.
- Recovery resulted in complete immunity.

6.2.1.1 The model

The state variables were the number of flying foxes each week that were:

S Susceptible to infection (but not yet infected).

I Infectious (and capable of infecting others).

R Recovered from infection.

N = S + I + R

The average weekly transition parameters were:

i the rate of transmission (the rate of transition from Susceptible to Infectious).

r the rate of recovery (the rate of transition from Infectious to Recovered).

The model (illustrated in Figure 6.1) was described by the following set of equations. The subscript $_t$ denotes time in weeks, with $_{t+1}$ being one week later:

Figure 6.1: Model 1 - A simple state-transition model for a hypothetical population of flying foxes where infection results in complete immunity.

$$S_{t+1} = S_t - iS_t I_t/N_t \quad (1)$$

$$I_{t+1} = I_t + iS_t I_t/N_t - rI_t \quad (2)$$

$$R_{t+1} = R_t + rI_t \quad (3)$$

Thus, in equation 1, the number of susceptible animals at the beginning of next week (S_{t+1}) equals the number susceptible at the beginning of this week (S_t) minus the number that become infected this week (iS_tI_t/N_t). The Microsoft™ Excel™ spreadsheet was used to provide numeric solutions to the equations and thus the model outputs. The outputs are primarily presented graphically.

6.2.1.2 Estimating model parameters

The rate of transmission (*i*) was defined as the average number of effective contacts per infectious flying fox per week. Thus, a transmission rate of 1 means that there was one contact sufficient to transmit infection (to a susceptible flying fox) per infectious flying fox per week. Rates of 0.5, 1, and 3 were used in the model. The rate of recovery (*r*) was defined as the inverse of the infectious period. The rates used in the model were 0.2,

(corresponding to infected animals being infectious for 5 weeks) 0.4 (corresponding to infected animals being infectious for 2.5 weeks) and 0.8 (corresponding to infected animals being infectious for 1.25 weeks). Experimental studies indicate that seroconversion in flying foxes occurs between days 14 and 21 post-inoculation (Williamson *et al.*, 2000; Williamson *et al.*, 1998), which supports the higher rates of recovery (assuming infectiousness ceases with seroconversion).

6.2.1.3 Model verification and validation

The model was tested over a range of scenarios to verify its structure and mathematical soundness. The model outputs were checked for consistency with qualitative observations of the infection dynamics in flying foxes, although lack of knowledge of the actual behaviour of HeV infection in flying fox populations precluded direct validation of the outputs.

6.2.1.4 Model experiments

A trial and error method was used to investigate the behaviour of infection in the hypothetical population. The model was consecutively run with the various parameter combinations to determine which parameters controlled the rate of spread of infection. Population size (N=10,000 and N=500,000) and the number of infectious animals initially introduced to the population (n=1 and n=100) were also varied to establish their effect on infection dynamics.

6.2.2 Model 2

Model 2 is an extension of Model 1. The following assumptions were made:

- The size of the population was constant, but included births and deaths at equal rates.
- Infection was directly transmitted.
- All flying foxes were equal with respect to susceptibility and infectivity.
- Following infection, each flying fox was uniformly infectious throughout its infectious period.
- Births were pulsed annually.
- All births were susceptible to infection.
- Deaths occurred at a constant rate.
- Infection did not result in any change in the rate at which deaths occurred.
- Infection resulted in complete immunity.

6.2.2.1 The model

The state variables were the number of flying foxes each week that were:

S Susceptible to infection.

I Infectious.

R Recovered from infection.

B Born into the population.

D Died.

N = S + I + R + B - D

The average weekly transition parameters were:

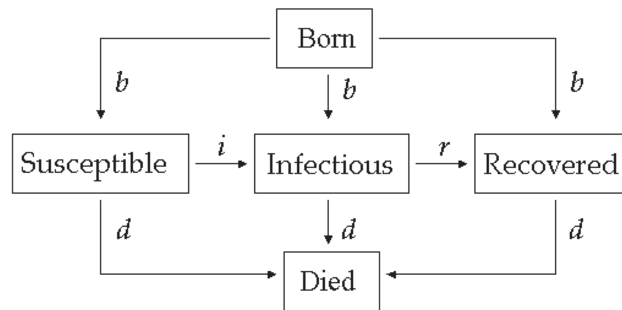
i the rate of transmission (the rate of transition from Susceptible to Infectious).

r the rate of recovery (the rate of transition from Infectious to Recovered).

d the rate of death of flying foxes.

b the rate of birth of flying foxes (equal to *d*).

Figure 6.2: Model 2 – A simple state-transition model for a hypothetical population of flying foxes incorporating births and deaths at equal rates.



The model (illustrate in Figure 6.2) was described by the following set of equations. The subscript $_t$ denotes time in weeks, with $_{t+1}$ being one week later:

$$S_{t+1} = S_t - iS_t I_t/N_t + b(S_t + I_t + R_t) - dS_t \qquad (4)$$

$$I_{t+1} = I_t + iS_t I_t/N_t - rI_t - dI_t \qquad (5)$$

$$R_{t+1} = R_t + rI_t - dR_t \qquad (6)$$

$$B_{t+1} = B_t + b(S_t + I_t + R_t) \qquad (7)$$

$$D_{t+1} = D_t + d(S_t + I_t + R_t) \qquad (8)$$

The Microsoft™ Excel™ spreadsheet was again used to provide numeric solutions to the equations and thus the model outputs.

6.2.2.2 Estimating model parameters

The rates of transmission (i) and recovery (r) are as described for Model 1. The annual birth rate (b) was defined as the proportion of the population borne each year. The annual death rate (d) was defined as the proportion of the population that died each year. An assumed annual rate of 20% (equivalent to a weekly rate of 0.004) was used for b and d. This is consistent with estimates of fecundity and mortality by McIllwee & Martin (2002). The annual birth cohort was modelled as a pulse in weeks 49-52 each year, reflecting the typical seasonal breeding pattern in Australian flying foxes (Hall & Richards, 2000; McIllwee & Martin, 2002). The death rate was assumed constant over the year.

6.2.2.3 Model verification and validation

As described in Section 6.2.1.3.

6.2.2.4 Model experiments

The model was again consecutively run with various parameter combinations, population sizes of 10,000 and 500,000, and one initially infected animal. Values of 0 and 0.004 were used for the parameters b and d. A trial and error method was used to determine which parameters most influenced the persistence of infection in the population.

6.3 Results

6.3.1 Model 1

The model was sequentially run with the various parameter combinations and N=10,000. With i=0.5 and r=0.4, a protracted but minor outbreak (duration 151 weeks) was seen. The majority of susceptible animals remained unaffected (Figure 6.3). With i=1 and r=0.4, an early moderate outbreak was seen (duration 44 weeks), and most susceptible animals were infected (Figure 6.4). With i=3 and r=0.4, an early major outbreak occurred (duration 26 weeks) and all susceptible animals were infected (Figure 6.5). When the rate of recovery was decreased (r=0.2), the speed and the size of the outbreak increased. When the rate of recovery was increased (r=0.8), the converse occurred. No outbreak occurred with i=0.5 and r=0.8.

Increased population size (N=500,000) was associated with an increased outbreak size and duration. Outbreak duration was maximized at i=0.5 and r=0.4. An increased number of infectious animals initially introduced (n=100) was associated with an increased speed of outbreak onset, but had little or no effect on outbreak size or duration.

6.3.2 Model 2

The model was run with the various parameter combinations, initially with N=10,000 and *b* and *d*=0. Infection persisted as a single outbreak beyond 52 weeks with *i*=0.5 (*r*=0.2 and 0.4) and *i*=1 (*r*=0.2, 0.4, and 0.8), but not *i*=3. With N=500,000, infection persisted beyond 52 weeks for all tested levels of *i*, although only at the slowest rate of recovery (*r*=0.2) with *i*=3. Persistence was maximized at *i*=0.5 and *r*=0.4 regardless of population size.

With N=10,000, *b* and *d*=0.004, *i*=0.5 and *r*=0.4, infection persisted to 311 weeks as a protracted minor outbreak (Figure 6.6). With N=500,000, infection persisted beyond 520 weeks with a decreasing oscillatory pattern following a 40-60 week cycle (Figure 6.7).

6.4 Discussion

It is evident from the Model 1 outputs that the rate of transmission and the rate of recovery are the key parameters determining the rate of spread of infection in the hypothetical population. An increase in the rate of infection resulted in an increase in the size of the outbreak, regardless of other parameters. Similarly, a decrease in the rate of recovery (that is, an increase in the period of infectiousness) resulted in an increase in the size of the outbreak. These observations are intuitive, and the model outputs are consistent with state-transition modelling theory, in that the size and duration of simulated outbreaks is determined primarily by the rate of transmission (*i*) and the rate of recovery (*r*). The average number of effective contacts of an infectious animal with any other animal is represented by *i/r*, known as the basic reproductive number R_o (R nought). When R_o is greater than 1, the probability of a major outbreak is higher; when R_o is less than 1, the outbreak will be non-existent or minor (Graat & Frankena, 2001).

The Model 1 outputs also showed that increasing the population size increased the duration of the outbreak. Intuitively, increasing the size of the population will increase the number of susceptible animals and so prolong the period of effective contact, allowing infection to persist in the population for longer. Indeed the concept of a threshold susceptible population is central to ecological epidemiology (Swinton *et al.*, 1998), and a number of authors have reported the importance of population size in the persistence of morbillivirus infections (in Bolker and Grenfell (1995)). Swinton *et al* (1998) contend that high levels of transmission between harbour seal sub-populations, combined with the small annual birth cohort, make Phocine distemper virus persistence impossible in the

limited North Sea population. Bolker and Grenfell (1995) and others contend that measles can only persist in human communities larger than 250,000-500,000 people, because of the inherent high rate of transmission and recovery. They associate measles persistence with population heterogeneity and population spatial structure. The former encompasses factors such as age structure, social structure and genetics, and argues that infection can persist by, for example, affecting different community groups in different years; the latter encompasses metapopulation theory, whereby infection persists via spatially discrete 'patches' in which infection dies out sequentially rather than simultaneously. Swinton *et al* (1998) also discuss persistence thresholds in the context of metapopulations, and propose three determinants of critical community size: demographic parameters controlling the birth of susceptibles, epidemic parameters controlling the spread within sub-populations (patches) of the total population, and spatial parameters controlling the spatial structuring of the epidemic. They argue that at low levels of transmission, at weaker levels of spatial coupling, and at low levels of recruitment, critical community size is largely determined by the spatial structure of the population. That is, the population needs enough patches of sufficient size to maintain infection across the metapopulation while ensuring its absence from each patch long enough for the number of susceptibles to replenish. Where there are high rates of transmission, strong levels of spatial coupling, and high levels of recruitment, the authors contend it is the timing of the susceptible recruitment (and thus the demographic parameters) that is most important. By illustration, their deterministic modelling of PDV in North Sea harbour seals suggests that infection could only persist in a particular patch if the birth cohort was more than (an improbable) 35% of the population, and if infection were absent from each patch for several years. In Australia, most populations of flying foxes breed seasonally, so recruitment of susceptible animals from annual birth cohorts is typically clustered annually. Therefore, infection in any given population patch needs to persist for a year to take advantage of these new susceptibles. Model 1 suggests that persistence is dependent on the rate of transmission and population size. In a population of 500,000, infection persisted beyond 52 weeks for all tested levels of i (although only at the slowest rate of recovery with i=3). In a population of 10,000 however, infection only persisted beyond 52 weeks at i=0.5 and 1. Thus, consistent with above discussion, the model suggests that the rate of transmission and patch size are key factors if indeed Hendra virus infection is maintained in a spatially structured metapopulation.

The simulated effect of population size on the dynamics of infection raises an issue fundamental to validity of this type of ecological modelling. Is R_0 constant for all population sizes? Intuitively, a larger population means the probability of contact with a particular individual is decreased. Swinton *et al* (1998) contend that the way the population responds to an increase in size is fundamental. For example, where transmission is density-dependent, whether density changes with population size; where the population is spatially structured into patches, whether additional patches develop with increasing population size. There is discussion in the literature on whether infection transmission best follows 'true' mass-action theory or so-called 'pseudo' mass-action theory (de Jong, 1995; McCallum *et al.*, 2001). The former assumes that the density of all groups (susceptible, infectious and recovered) is constant regardless of population size, with the transmission rate represented by $\beta SI/N$; the latter does not, the transmission rate being represented by βSI. The modelling in this chapter follows true mass action theory, and thus R_0 is regarded as independent of population size.

The model 2 outputs are not consistent with long-term maintenance of infection. In a population size of 10,000 with an annual birth/death rate of 20%, infection will only persist in stable oscillatory pattern when the rate of recovery is an implausible 0.1 or lower. That is, when the period of infectiousness is 10 weeks. Similarly, in a population size of 500,000, infection only persists in stable pattern when r=0.2 or lower. Infection failed to persist at levels of r indicated by experimental studies (0.4-0.8), even at low rates of transmission. The availability of susceptible animals is the primary factor limiting persistence. Thus, persistence of infection at the level of r indicated by the experimental studies is inconsistent with the model assumption of lifelong immunity following infection and recovery. A second model assumption – that the population is closed - may also limit the number of susceptible animals in the population, although the immigrant ratio of susceptible to recovered animals would need to be implausibly high and regular to impact long-term persistence. Notwithstanding that the preceding discussion has discounted the likelihood of an extended period of infectiousness, a pattern of latency and recrudescence associated with vertical transmission (as proposed in Chapter 4) might promote such a pattern, although it is improbable that such a scenario would be sufficient to maintain infection in the absence of increased proportion of susceptible animals in the population. Further, an extended period of infectiousness is inconsistent with the reported difficulty in recovering virus from naturally infected flying foxes (Halpin *et al.*, 2000; Field HE, unpubl. data). It is also arguable that the simplifying assumption of an equal birth and

death rate might compromise the model's performance. However historic evidence indicates a substantial decrease in the size of the flying fox population in eastern Australia this century (Hall & Richards, 2000), suggesting that the assumption is conservative.

Model 1 illustrates the effect of the rate of transmission on the pattern of the outbreak and on the number of susceptibles remaining after the outbreak. As the rate of transmission increased, the number of susceptibles remaining after the outbreak decreased, reaching zero with i=3. The seroprevalences reported in Chapter 3 (ranging from 14% for *P. scapulatus* to 56% for *P. alecto*) suggest a rate of transmission between 0.5 and 1. According to Swinton *et al* (1998), this scenario better supports a critical community size 'patch' theory of persistence. Additional support for this hypothesis is provided by the apparent mature relationship between HeV and flying foxes in Australia described in Chapter 3. The phylogenetic distance of viruses of the genus *Henipavirus* from other viruses in the family *Paramyxoviridae* suggests that these are ancient viruses that likely have an evolutionary association with their hosts. Such an association strengthens the biological plausibility of maintenance of infection in flying foxes based on the spatially heterogenous population structure and the nomadic nature of the host species. The primary driver of nomadic movements in flying foxes is food resource, and specialist species such as *P. poliocephalus* and *P. scapulatus* appear particularly dynamic. However data on flying fox population dynamics is lacking, and meaningful estimates of the strength of spatial coupling between patches are impossible. Limited data on the level and frequency of interaction between species also limits discussion of whether a particular species may play a central role in the maintenance of infection. Certainly sub-populations of *P. scapulatus* in northern Australia are reported to periodically coalesce into large groups of hundreds of thousands of animals before undertaking major nomadic forays (Hall & Richards, 2000). The scale of these movements and the associated coupling, and the potentially higher rates of susceptibility in *P. scapulatus* associated with the lower seroprevalence described in Chapter 3, suggest that the role of this species in the maintenance of Hendra virus warrants further investigation. This discussion is continued in Chapter 7.

A number of previously discussed findings suggest that vertical transmission and latency may be a feature of Hendra virus infection in flying foxes. While this modelling has not directly explored the effect of either on persistence, the model outputs suggest that the number of susceptible animals, rather than the number of infectious animals, is the

primary infection-limiting factor. Further, Model 1 suggests that increasing the number of infectious animals introduced to a population had little or no effect on the duration of infection. However, it is worth noting that Anderson and May (Waithman, 1979) comment that vertical transmission decreases the size of the threshold population needed for a successful introduction of infection, and, where the population is regulated by infection, that vertical transmission lowers the equilibrium population of the host. Thus, additional modelling to specifically investigate the effect of infectious individuals within the annual birth cohort is warranted.

6.5 Conclusions

It is evident from Model 1 that the rate of transmission and the rate of recovery are the key parameters determining the rate of spread of infection. It is further evident that population size is positively associated with outbreak size and duration, and that infection will not persist long-term in a population without replacement of susceptible animals. Further, it is evident from Model 2 that where birth is clustered annually, infection that fades out within a year cannot persist regardless of the birth rate. Thus, given the positive association between population size and outbreak duration, patch size is a key determinant of persistence in a spatially structured metapopulation. However, in this model, infection failed to persist at levels of r indicated by experimental studies, even at low rates of transmission, suggesting that persistence at these levels of recovery is inconsistent with the model assumption of lifelong immunity following infection and recovery. Notwithstanding, low rate of transmission suggested by the seroprevalences reported in Chapter 3 and the mature host-agent relationship support maintenance of infection in flying foxes based on the spatially heterogenous population structure and the nomadic nature of the species.

Further modelling is necessary to support these conclusions and to investigate other potentially important variables. In addition to vertical transmission and latency, non-heterogenous mixing within flying fox populations and non-linear transmission of infection need to be considered in seeking to build a model that best fits the observed dynamics of Hendra virus infection in flying foxes. Also, the effects of stochastic events and the effects of any between-year variability in births, deaths, immigration and emigration were not addressed here. It is likely that the more sophisticated stochastic compartmental model approach used by Swinton *et al* (1998) to investigate persistence thresholds for

Phocine distemper virus in harbour seal metapopulations could be usefully applied to flying foxes.

Figure 6.3 Model 1 output with N=10000, *i*=0.5, *r*=0.4.

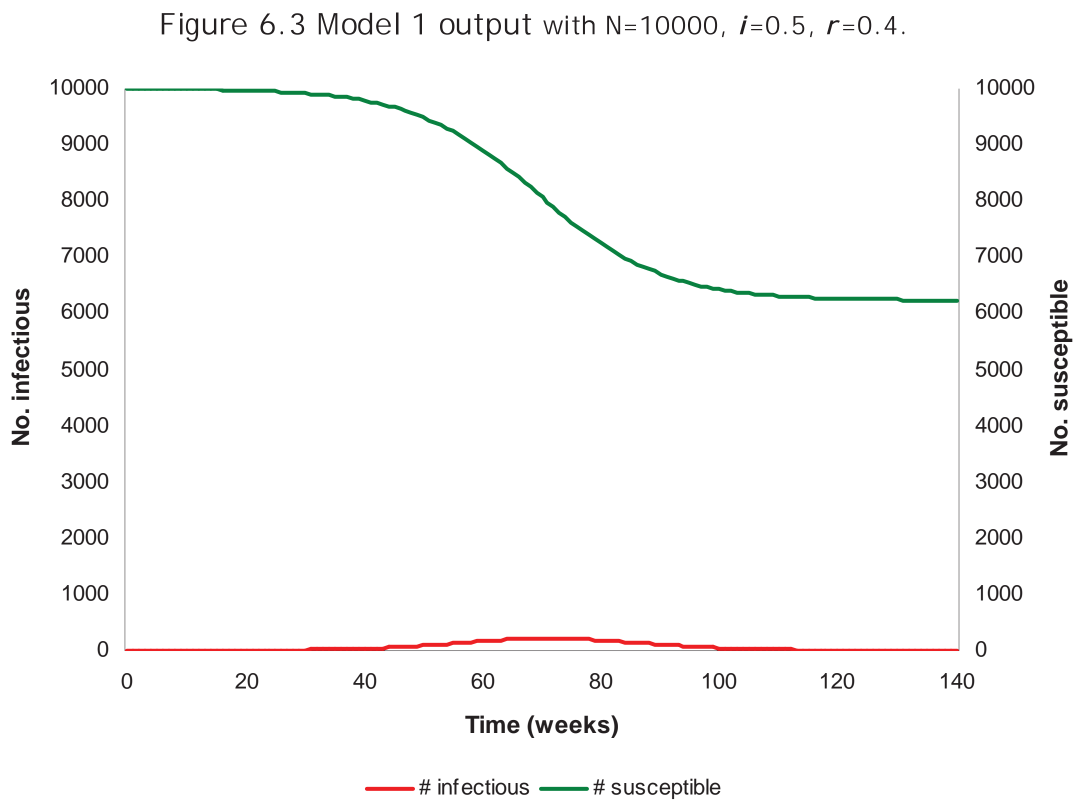


Figure 6.4: Model 1 output with N=10000, *i* =1, *r* =0.4.

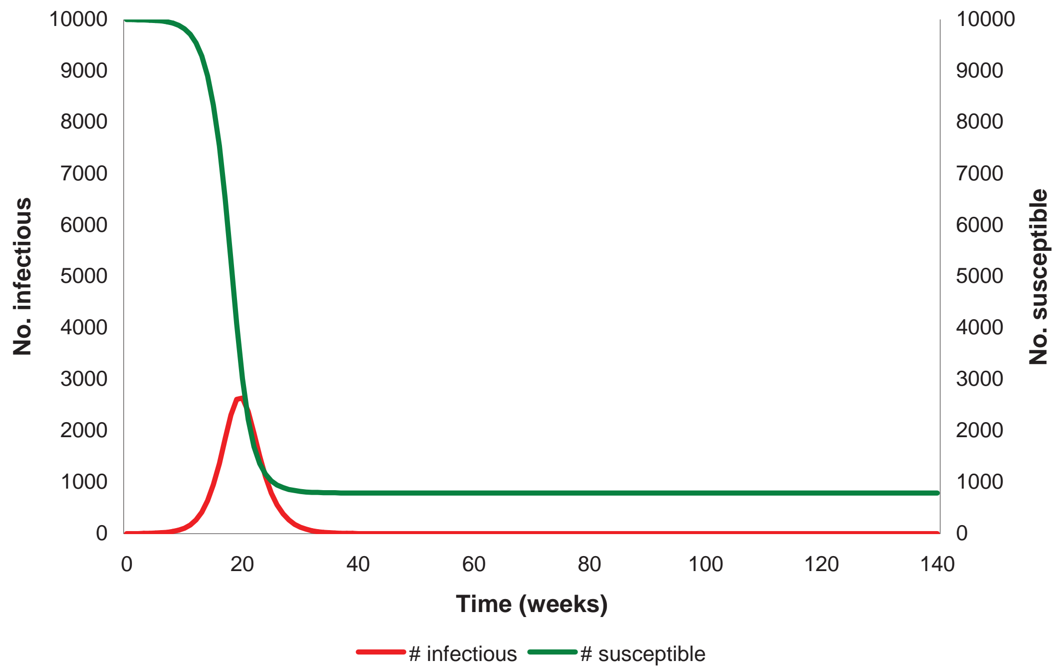


Figure 6.5: Model 1 output with N=10000, *i* =3, *r* =0.4.

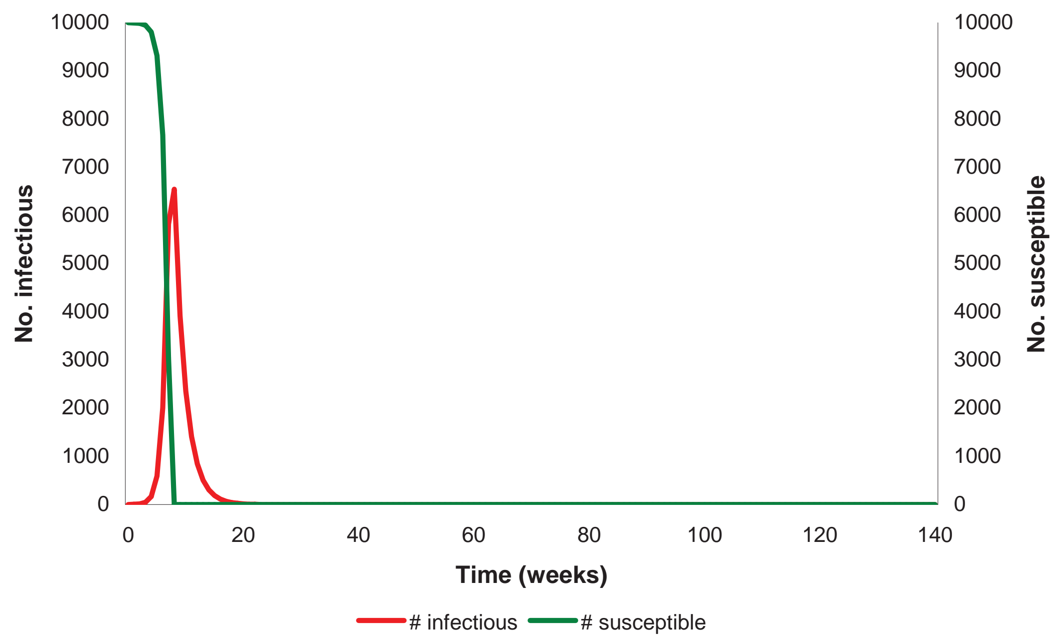


Figure 6.6: Model 2 output with N=10,000, *i*=0.5, *r*=0.4, *b* & *d*=0.004

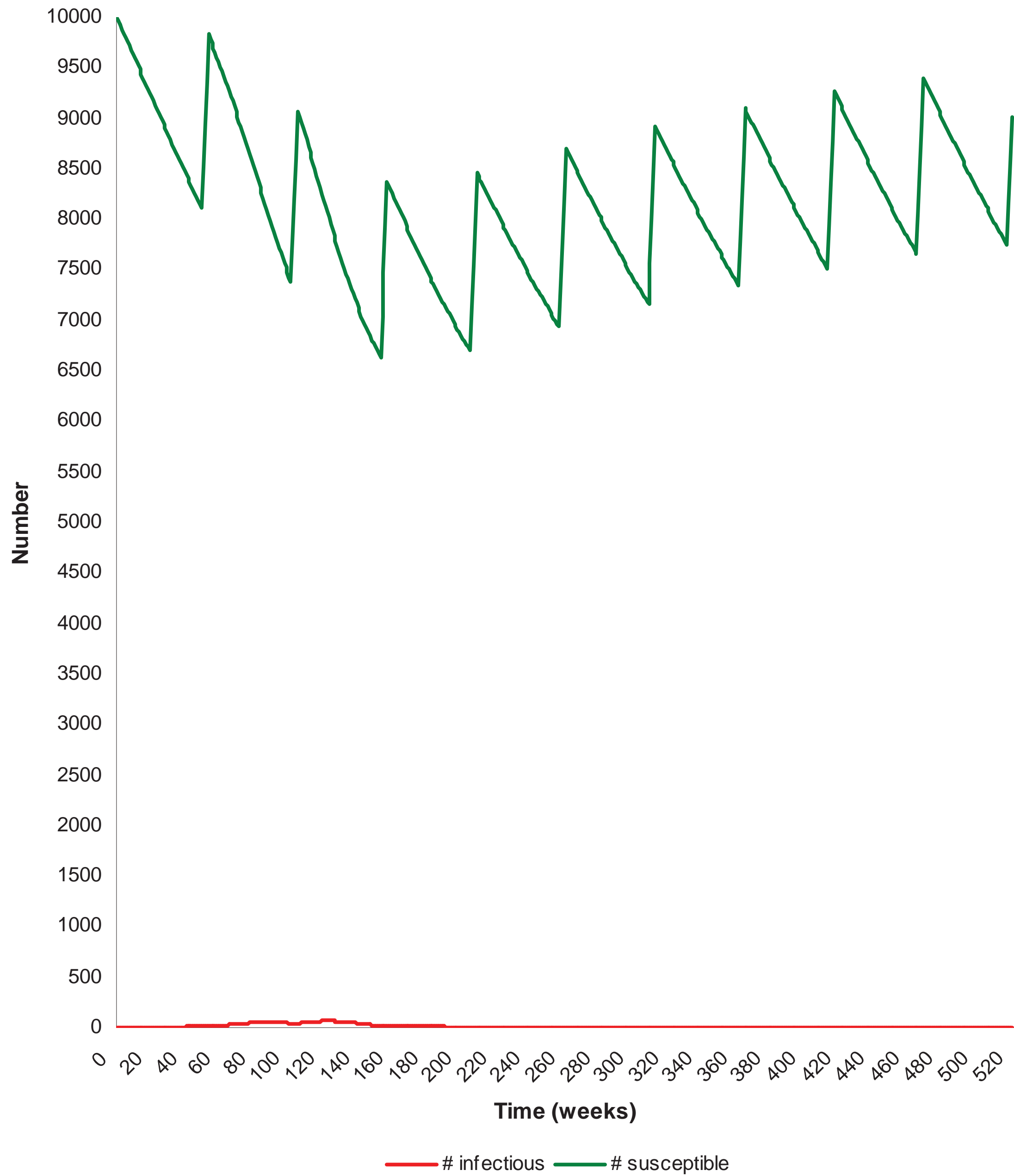
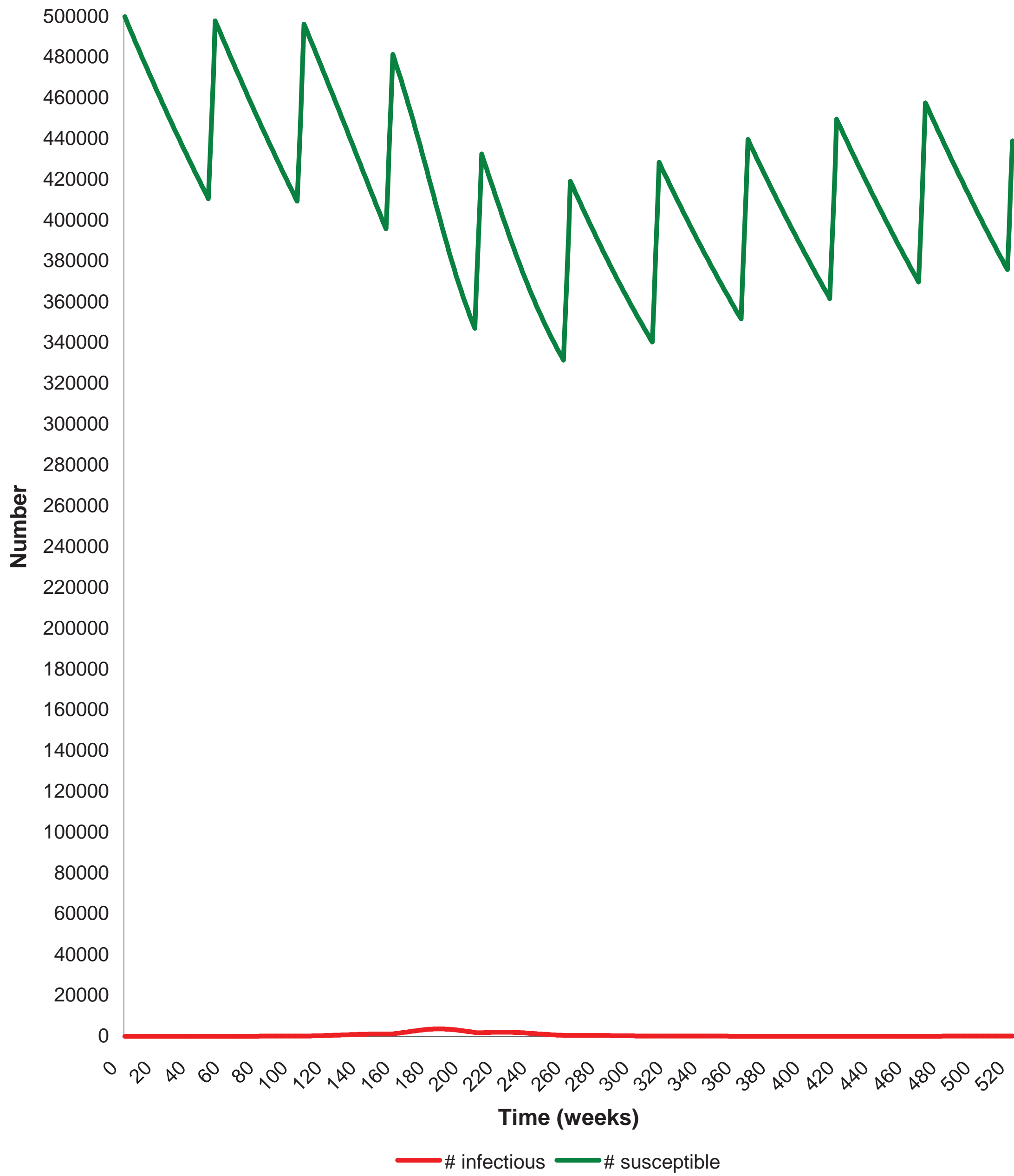


Figure 6.7: Model 2 output with N=500,000, *i*=0.5, *r*=0.4, *b & d*=0.004



CHAPTER 7

GENERAL DISCUSSION

7.1 Introduction

Chapter 3 described the widespread occurrence of Hendra virus infection in free-living Australian flying fox populations, identified risk factors for infection in flying foxes, and argued a mature host-virus relationship in the Australian landscape. Chapter 4 described longitudinal studies in a captive flying fox population and suggested latency, seasonal recrudescence and vertical transmission as features of Hendra virus infection in flying foxes. Chapter 5 described the wide geographic and taxonomic distribution of Australian bat lyssavirus infection in Australian bats, identified risk factors for infection, identified the occurrence of non-fatal infection in bats, and argued a mature relationship between ABLV and Australian bats. Chapter 6 described the modelling of Hendra virus infection in a hypothetical flying fox population and indicated that the rate of transmission, the rate of recovery, and patch size were key determinants in the maintenance of infection in flying foxes. This work has substantially advanced understanding of the origins of Hendra virus and ABLV in Australia, the ecology of infection of both agents in Australian bat, and risk factors for infection for both in bat and non-bat species. However, several important aspects of the ecology of infection, and factors of associated with spillover, remain poorly understood. How is Hendra virus infection transmitted in flying foxes? What is the mode of transmission to horses? What factors precipitated the emergence of Hendra virus and ABLV? This chapter will discuss these and other outstanding issues and propose plausible explanatory hypotheses.

7.2 Hendra virus

7.2.1 Transmission of infection in flying foxes

The study findings suggest that both horizontal and vertical transmission of Hendra virus may occur in flying foxes. In Chapter 4, the findings of both longitudinal studies suggest that the presence of infection in the captive colony, and the pattern of infection, is inconsistent with direct horizontal transmission. Firstly, the identification of a single viraemic flying fox and the absence of detected infection any in-contact flying foxes (Study 2) is better explained by recrudesence of a latent infection than by horizontal

transmission. Secondly, the association between dam and pup serostatus and the persistence of antibodies in pups, and the seasonal boost in antibody titres in mature age flying foxes (Study 3) in the absence of seroconversion in any in-contact flying foxes suggest vertical transmission. The findings of several other authors support a hypothesis of vertical transmission. A pregnant flying fox from which Halpin *et al* (2000) recovered virus in uterine fluids and foetal tissues was seropositive by ELISA and SNT, demonstrating that Hendra virus can persist in the presence of neutralising antibodies. Williamson *et al* (1998) similarly showed virus to persist in an experimentally infected horse in the presence of neutralising antibodies. Latency, recrudescence, and virus persistence in the presence of neutralising antibodies was also evident in the second human Hendra virus case, with a fulminating infection occurring 13 months after the original infection (O'Sullivan *et al.*, 1997). In addition, several epidemiological observations support an association between Hendra virus infection and pregnancy – at least two of the three index case horses were pregnant; there is a putative temporal association between spillover to horses and late pregnancy in flying foxes in at least two of the three spillovers; and experimental studies in two species (flying foxes and guinea pigs) suggest a virus predilection for the placenta. Thus, a plausible hypothesis is that vertical transmission of Hendra virus infection in flying foxes is effected by the recrudescence of virus latent in the uterus of pregnant flying foxes, and that in-utero infection of the foetus occurs as a result of transplacental infection.

However, it is evident that vertical transmission is not the sole mode of transmission in flying foxes (and it is obviously not the mode of transmission to horses). As previously stated, persistent antibody titres in mature male flying foxes in Chapter 4 (Study 3), the presence of an association between age and HeV serostatus and the absence of an association between sex and HeV serostatus (Chapter 3) are inconsistent with vertical transmission. However, routes of excretion of Hendra virus in flying foxes were not identified in Chapter 4. Cell culture of throat and urogenital swabs failed to detect virus in the single viraemic flying fox identified in Study 2. This animal was negative for virus and for neutralising antibodies at preceding weekly samplings, and at the two subsequent weekly samplings, after which she was euthanased. No virus was recovered from tissues at necropsy. Initial experimental infections in flying foxes also failed to identify possible routes of virus excretion. Tissue, urine and faecal samples from *P. poliocephalus* infected with 50,000 $TCID_{50}$ were all negative by cell culture (Williamson *et al.*, 1998). However, virus isolation from two naturally infected flying foxes described by Halpin *et al* (2000)

indicates one putative route of excretion in flying foxes – foetal tissues or fluids. The authors isolated virus from uterine fluid from a pregnant *P. poliocephalus*, from a pooled sample of the liver and lung of her aborted foetuses, and from the lung of the foetus of a second (*P. alecto*) flying fox. Later experimental infections in pregnant *P. poliocephalus* also support foetal tissues as a putative route of excretion (Williamson *et al.*, 2000). Placental tissues from flying foxes killed ten days post-inoculation were positive by immunohistochemistry. Virus was also recovered from kidney, blood, heart and spleen at ten days post-inoculation, suggesting urine and blood as possible routes of excretion (although virus was not recovered from the urine, mouth, nose or rectum of the infected animals). The isolation of Nipah virus from the urine of naturally infected *P. hypomelanus* in Malaysia (Chua *et al.*, 2002) also supports urine as a putative route of excretion for the closely related Hendra virus. Certainly, within flying fox camps, lower roosting individuals are regularly exposed to the urine of higher roosting individuals. This, in conjunction with the practice of flying foxes using urine as an aid to grooming (LS Hall, School of Veterinary Science, UQ; pers. comm.), supports infection via the oral route from the ingestion of infected urine as a plausible mode of horizontal transmission of Hendra virus infection in flying foxes. The isolation of Nipah virus from fruit partially eaten by a flying fox (Chua *et al.*, 2002) suggests that saliva is a route of excretion for that virus, and so plausibly for Hendra virus. However, while Williamson *et al* (1998) recovered virus from the saliva of experimentally infected horses, none was recovered from similarly infected cats, guinea pigs or flying foxes.

The possibility of indirect transmission of Hendra virus raised in Chapter 3 warrants further discussion here. To date, two groups of insects have been investigated - mosquitoes and nycteribid flies (See Chapter 3). Nycteribids (*Cyclopodia albertisii*, family *Nycteribiidae*) are obligate blood-sucking ectoparasites of flying foxes. While insect-vectored transmission is unknown in viruses of the family *Paramyxoviridae*, both mosquitoes and nycteribids could plausibly mechanically transmit infection between flying foxes, and mosquitoes could plausibly likewise transmit infection from flying foxes to horses. No evidence of infection was found in pooled non-random (convenience) samples of nycteribids from multiple locations and times. However a Hendra virus PCR product was obtained from a pooled sample of mosquitoes from the Brisbane index case paddock at Cannon Hill in 1995(AR Gould, AAHL; pers. comm.). Subsequent screening of mosquitoes at multiple locations and times failed to identify any other positive samples. Thus, interpretation of the positive Cannon Hill PCR should consider the likely low positive

predictive value of the test and the possibility that this was a false positive result. Nonetheless, the design of both studies constrains interpretation and it is inappropriate to conclude that neither species plays a role. A key issue is the infection status of the flying foxes on which sampled insects were feeding. It is implausible that blood-feeding insects feeding on non-viraemic flying foxes could be positive, thus, unless insects are feeding on known positive flying foxes, negative results cannot be meaningfully interpreted. Experimental studies could readily test a hypothesized role of biting insects in the transmission of Hendra virus.

7.2.2 Transmission of infection to horses

The mode of transmission of Hendra virus infection to horses has yet to be established. However, epidemiological investigations of natural infections in horses and flying foxes, and the outcomes of experimental infections in a range of species, provide useful information. Firstly, respiratory spread has not been demonstrated experimentally in any species, and the spatial pattern in naturally infected horses has not been consistent with respiratory spread. Secondly, Hendra virus has been isolated from the kidney and urine of horses and cats experimentally infected with Hendra virus, and cat-to-cat transmission and suspected cat-to-horse transmission have been attributed to exposure to infected urine (Westbury *et al.*, 1996; Williamson *et al.*, 1998). Thirdly, horses have been experimentally infected by the naso-oral route (Williamson *et al.*, 1998).

Thus, hypotheses involving (i) the excretion of an infective dose of Hendra virus from a flying fox, (ii) contamination of pasture, and (iii) ingestion of the contaminated pasture by a susceptible horse are plausible. Young *et al* (1997) proposed that transmission from flying foxes to horses was effected by contact with infected foetal tissues or fluids via the ingestion of recently contaminated pasture. This hypothesis was largely based on the August-September temporal overlay of the Brisbane and Mackay spillovers with the late gestation of *P. alecto* and *P. poliocephalus* in Queensland, and on the absence of evidence of infection in flying fox carers regularly exposed to other potential routes of excretion such as urine, faeces and oro-nasal secretions. Notwithstanding the latter, an alternative hypothesis is that the ingestion of pasture contaminated with infected flying fox urine is the mode of transmission to horses. Although Hendra virus is yet to be isolated from flying fox urine, the previously described isolation of Nipah virus from the urine of free-living Malaysian flying foxes (Chua *et al.*, 2002) supports urine as a putative route of excretion for Hendra virus. The urine hypothesis is also supported by the experimental studies

described above that attribute cat-to-cat and probable cat-to-horse transmission to exposure to infected urine. Another plausible hypothesis is that the ingestion of masticated fruit dropped by feeding flying foxes is the mode of transmission to horses. The quantity of these 'spats' under food trees bearing fibrous fruit (such as the *Ficus* species present in the Brisbane index case paddock at Cannon Hill) can be substantial, and they may represent an attractive source of saliva-laden virus to grazing horses. Certainly feral pigs have been observed to readily consume spats (LS Hall, School of Veterinary Science, UQ, pers. comm.). The viability of virus in spats is also likely to be prolonged due to slowed desiccation, heat and ultraviolet action. As previously noted, Hendra virus has been isolated from the oral cavity of experimentally infected horses (Williamson *et al.*, 1998) and the closely related Nipah virus has been isolated from fruit partially eaten by a flying fox (Chua *et al.*, 2002), supporting saliva as a plausible route of excretion of Hendra virus from flying foxes.

It should be recognized that the mode of transmission between flying foxes and the mode of transmission from flying foxes to horses may differ. The infectious dose, the routes of infection, and the physiological risk factors for infection in both species are unknown or incompletely understood. As previously discussed, there is some evidence to suggest that pregnancy is a risk factor for infection in both flying foxes and horses. Managing the risk of spillover to horses is further constrained by the lack of knowledge of the incidence of infection and the temporal pattern of infection (and thus excretion) in flying foxes. Regardless of the mode of transmission to horses, it is evident from natural infections and experimental studies that horse-to-horse transmission is not readily effected. The apparent exception is the first recognized outbreak in the Hendra stables that involved 21 equine cases. However the temporal pattern of infection in this outbreak suggests that the index case was the source of infection for all cases and that no secondary infection occurred (Baldock *et al.*, 1996). Indeed, it is probable that horse-to-horse transmission in this instance was inadvertently facilitated by husbandry practices or other actions in the stable that resulted in the direct transmission of infected body fluids. Two plausible scenarios have been proposed – that a common syringe and needle was used to administer medication to the index case and to other (subsequently infected) horses; or that a cloth, bridle or other piece of equipment contaminated with infectious oral secretions from one horse was used on other (subsequently infected) horses. Likewise it is evident that horse-to-human transmission does not readily occur. Many people were potentially exposed to infection in the investigation of the Brisbane outbreak in particular, yet only the trainer

and a strapper succumbed - both were closely involved in nursing the index case. Whether the apparent low infectivity for horses and humans is a reflection of the innate infectivity of Hendra virus, the instability of the virus outside the host, or of ineffective contact is unclear, although experimental studies support the former.

7.2.3 Maintenance of infection in flying foxes

Chapter 3 describes a spatial and temporal pattern of infection in Australian flying fox species that is consistent with endemicity. It is evident that the virus is not recently introduced to Australia but maintained in a wildlife reservoir from which it emerged. The mechanism of maintenance in flying foxes is fundamental to managing the risk of future spillovers and to understanding factors that precipitated emergence. (The latter is discussed in Section 7.4). Haydon *et al* (2002) define a reservoir as 'one or more epidemiologically connected populations or environments in which the pathogen can be permanently maintained, and from which infection is transmitted to the defined target population'. This definition is attractive because it recognizes that a discrete animal population or an ecological community can constitute a reservoir. It also emphasizes the permanent maintenance of infection.

There are three plausible hypotheses for the maintenance of Hendra virus infection in flying foxes. Either infection is maintained in a single panmyctic population, or secondly, in a spatial, temporal and species mosaic across within a metapopulation, or thirdly, in a discrete subset of the metapopulation, with a pulsing epidemic pattern in the balance of the metapopulation. The latter is the preferred hypothesis – specifically, that infection permanently maintains in *P. scapulatus*, and periodically spills over into *P. alecto*. *P. poliocephalus* and *P. conspicillatus*. The following arguments support this hypothesis. Firstly, the modelling suggests that *P. scapulatus* may better maintain infection than the other species. The models indicate that infection will more readily persist in a population if the rate of antibody loss is high (consistent with a low seroprevalence) and the patch size larger. Secondly, the biology of *P. scapulatus* differs from the other species in several respects including colony size and density. In northern Australia, *P. scapulatus* typically exist in colonies of tens of thousands or hundreds of thousands of animals. Roosting density is also greater than other species, with animals typically roosting in dense clusters of 20 or 30. Population size has been identified as a key factor in the maintenance of other (directly transmitted) paramyxovirus infections including measles (Bolker & Grenfell, 1995) and phocine distemper virus (Swinton *et al.*, 1998). Thirdly, the movement

patterns of *P. scapulatus* are more extensive than other species. *P. scapulatus* is highly mobile, and coalesced populations periodically make major nomadic movements following seasonal food resources[50]. During these movements, they use 'local' flying fox camps as roosts, typically swelling numbers from thousands to tens or hundreds of thousands of flying foxes, with a resultant increase in density and physical interaction. In addition, a trend of increasing urbanization of flying foxes (including *P. scapulatus*) in eastern Australia has been observed throughout the 1980s and 1990s. This has been attributed to a reduced availability or suitability of historic sites because of land-use changes, and a concurrent increase of reliable food sources in well-watered suburban gardens (Hall & Richards, 2000; Parry-Jones & Augee, 2001). This latter point is elaborated in the next section. Thus it is contended that Hendra virus infection is permanently maintained in the large susceptible populations of *P. scapulatus* in northern Australia, and that populations of *P. alecto*. *P. poliocephalus* and *P. conspicillatus* are subject to a pulsing epidemic pattern of infection governed by the susceptible threshold in these populations and periodic exposure to the nomadic *P. scapulatus*.

7.3 Australian bat lyssavirus

7.3.1 The transmission and maintenance of ABLV in flying foxes

Chapter 5 describes a pattern of infection in Australian bats (both mega- and microchiroptera) that is consistent with endemicity. As with Hendra virus, the findings support the recent emergence of ABLV, rather than its recent introduction to Australia. Again as with Hendra virus, an understanding of the mechanism of maintenance of ABLV infection in Australian bat populations is fundamental to managing the risk of future spillovers and to understanding factors that precipitated emergence.

Unlike the novel Hendra virus, understanding of the transmission (and to a lesser extent, maintenance) of ABLV has been considerably aided by studies of lyssaviral infections elsewhere. It is well established that lyssaviral infections are primarily transmitted by infected saliva transdermally or transmucosally. Reports of transmission associated with respiratory secretions are rare and limited to cave-dwelling species (Constantine, 1962; Constantine *et al.*, 1972), and the recent phenomenon in the USA of bat-variant human

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[50] It is also plausible that on occasion the (more limited) nomadic movements of *P. alecto*. *P. poliocephalus* and *P. conspicillatus* may either introduce infection to neighbouring colonies, or (depending on their HeV serostatus) increase the proportion of susceptible individuals in these colonies, and thus support the temporary maintenance of infection at a regional level.

rabies cases with no reported history of a bite or other direct bat contact is most plausibly explained by undetected bat bites (McColl *et al.*, 2000). Thus it is highly probable that ABLV is also primarily transmitted by infected saliva. Certainly this putative route of infection is consistent with the history of the two human cases of ABLV to date, in that both cases are reported to have had direct bat contact involving a bite. There are three points of particular interest in relation to the epidemiology of these cases – the temporal and spatial clustering of the attributed exposures, the incubation period, and the bat species involved. Against a background of no previously identified human cases of ABLV (notwithstanding the extensive overlap of bat and human distributions in Australia), both human infections occurred within several weeks and 500 km of each other. Further, these cases were attributed to bats from two different sub-orders. In the first reported case (in Rockhampton in central Queensland), infection was retrospectively attributed to the bite of *S. flaviventris* (a microchiropteran) that the wildlife carer had been rehabilitating (Gould *et al.*, 2002). The bite occurred 6 weeks before the onset of symptoms (Speare *et al.*, 1997), making the attributed exposure mid-September 1996. In the second case (in Mackay in central Queensland), the only history of contact with bats was in late August 1996, 27 months prior to the onset of symptoms, when the person was bitten by a flying fox (a megachiropteran) that she was removing from a child[51]. Both chance and the recent introduction of ABLV are improbable explanations for the clustered exposures. A plausible alternative is an increased incidence of ABLV infection in both mega- and microchiropteran populations in central Queensland at the time. The latter is consistent with a pulsing epidemic pattern of infection in bats, governed by intrinsic factors such as susceptibility threshold and by extrinsic factors such as seasonal immigration movements promoting exposure in local bat populations. When one also considers that *Species* is also an important animal-level risk factor for ABLV infection in bats (see Chapter 5), the hypothesis posed for HeV, of infection maintained in a subset (or subsets) of the Australian bat metapopulation from where it ignites periodic outbreaks in susceptible sub-populations, is also attractive for ABLV. The Chapter 5 findings support these subsets being *P. scapulatus* and *S. flaviventris*. *P. scapulatus* exhibited a higher antigen and antibody prevalence than other flying fox species, and the higher probability of infection in all species in the first two quarters is also consistent with an association between the nomadic movements of *P. scapulatus* and the incidence of ABLV infection. *S. flaviventris* was the only microchiropteran species in which antigen was detected, and it had a

[51] Details of the two human cases are presented in the literature review (Chapter 2).

significantly higher antibody prevalence than other species. The intense IHC staining (ie virus load) in the absence of lesions in *S. flaviventris* (Hooper *et al.*, 1999) supports adaptation of this species to ABLV and further argues for its reservoir host status. These findings suggest an important role for these species in the ecology of ABLV.

Data on the incubation period of ABLV in flying foxes is available from observations of natural infections and from experimental studies. Field *et al* (1999) reported a probable 6-9 week incubation in a hand-raised orphaned free-living *P. alecto*, and Warrilow *et al* (2003) reported a probable 4-week incubation in a *P. poliocephalus* in a captive colony. Incubation period in experimental studies (see Chapter 2) ranged from 10-19 days when *P. poliocephalus* were inoculated intramuscularly at four sites with $10^{5.2}$ - $10^{5.5}$ $MICED_{50}$ of a salivary gland preparation derived from a naturally infected flying fox (Barrett, 2004) to 14-27 days when *P. poliocephalus* were inoculated intramuscularly in the forelimb with $10^{3.7}$ or 10^{5} $TCID_{50}$ of single passage ABLV (McColl *et al.*, 2002). The nature of the lyssaviral infections (with virus traveling along peripheral nerves to the CNS) means that the incubation period primarily reflects the infection site, with bites distant to the CNS typically having a longer incubation period. In humans, the usual incubation period for rabies is 20-90 days, with 95% of cases occurring with a year of exposure (Hanna *et al.*, 2000). Extended incubations, such as the second human ABLV case may reflect delay in the virus entering the peripheral nervous system at the bite site.

As argued for Hendra virus, the pattern of ABLV infection in Australian bats better supports its recent emergence rather than its recent introduction to Australia. While the earliest report of confirmed infection[52] in Australian bats is January 1995, after Speare *et al* (1997) retrospectively identified infection in a *P. alecto* found behaving aggressively in a north Queensland backyard, the retrospective study in Chapter 5 presents histological findings that suggest ABLV may have been cycling in a north Queensland bat population in 1981. It is also possible that ABLV infection in flying foxes has previously been misdiagnosed. Indeed, Speare *et al* (1997) comment that the FAT-positive *P. alecto* referred to above also had histological and biochemical evidence of lead poisioning, and Sutton and Wilson (1983) described a clinical history consistent with ABLV in one of two cases of neurologic disease in flying foxes attributed to lead poisioning.

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[52] Confirmed by the gold standard FAT.

7.4 Factors associated with the emergence of Hendra virus and ABLV

Bats have a prominent role in the ecology of lyssaviruses. They are the exclusive or primary natural hosts of numerous rabies strains, European bat lyssavirus 1 and 2, Lagos bat virus, and Duvenhage virus, prompting a number of authors (McColl *et al.*, 2000; St. George, 1989) to suggest an evolutionary association between bats and lyssaviruses. While the latter is beyond the scope of this work, it is evident that bats have a prominent role in the ecology of ABLV as well, and that ABLV is not a recent introduction to Australia. Similarly, it is evident that Hendra virus is not a recent introduction. Thus how does one explain the absence of historic human infection, particularly given the exposure history of high-risk groups such as wildlife carers? Both chance and misdiagnosis are improbable explanations; the former because of the high probability of a case given the number of potential exposure events (Box 7.1); the latter because sporadic human viral encephalitides are sufficiently unusual in Australia to attract expert investigation. Enhanced surveillance certainly explains the identification of ABLV in bats, but not in humans. A more plausible explanation, and one common to infectious disease emergence, is a change in the host, agent or environment leading to an increased prevalence of infection in bats or an increased probability of human exposure. The temporally clustered emergence of not only Hendra virus and ABLV described in this thesis, but two other viruses, Menangle virus and Nipah virus (Box 7.2), argues for a host or environment-level change. Morse (1995), Krause (1992) and Lederberg *et al* (1992) propose that a series of commonly occurring anthropogenic environmental changes drive disease emergence by pushing pathogens outside their normal population parameters. They argue that the introduction of pathogens via global air travel and trade, the encroachment of human activities into wilderness regions, urbanization, climatic changes and agricultural intensification are common drivers of emergence. For zoonotic diseases with wildlife reservoirs, anthropogenic factors that alter wildlife population structure, migration patterns and behaviour may also drive emergence of disease in human populations (Daszak *et al.*, 2000; Daszak *et al.*, 2001). For example, human population encroachment into wildlife habitat may increase the risk of Lyme disease and other tick-borne encephalitides by driving the loss of less competent reservoir hosts (Ostfeld &

Box 7.1: Extrapolated at-risk exposures in bat carers
In the 10 years prior to the first description of ABLV in 1996, the southeast Queensland bat rehabilitation group ONARR (Orphan Native Animal Rear and Release) cared for an estimated 200-300 sick, injured or orphaned flying foxes per annum. Assuming the crude infection prevalence of 7.5% found in diagnostic sample in Chapter 5, the unvaccinated ONARR carers would have been exposed to 15-20 FAT-positive flying foxes annually.

Keesing, 2000). Likewise, the introduction of a "new" infection into a human or domestic animal population may follow the incursion of humans (accompanied by their domestic animals) into previously remote natural habitats where unknown disease agents exist in harmony with wild reservoir hosts. Upon contact with new species, an agent may jump species barriers, thereby spilling over into humans or livestock. Unlike the natural host, the new host may have no natural immunity or evolved resistance. Additionally, high population densities and management practices may facilitate the rapid spread of pathogens throughout livestock populations. Infection may be transmitted to humans directly from the natural host or via an intermediate (livestock) host (Field *et al.*, 2001).

Box 7.2: The emergence of Menangle and Nipah viruses.
Menangle virus (genus *Rubulavirus*, family *Paramyxoviridae*) was first described in 1997, causally associated with severe reproductive failure in a commercial piggery in New South Wales in Australia. The syndrome in pigs was one of mummified foetuses, stillbirths and foetal deformities (Philbey *et al.*, 1998). Two piggery workers suffered a severe febrile disease after exposure to infected pigs. Both had neutralising antibodies to Menangle virus (Chant *et al.*, 1998). Neutralising antibodies were found to be common and widespread in Australian flying foxes (Field, HE., unpubl. data)
Nipah virus (genus *Henipavirus*, family *Paramyxoviridae*) was first identified in 1999 as the primary aetiological agent in a major outbreak of disease in pigs and humans in Peninsular Malaysia (Chua *et al.*, 2000). Respiratory and neurological syndromes were seen in pigs (Nor *et al.*, 2000). The predominant clinical syndrome in humans was encephalitic (Chua *et al.*, 1999b). Of 265 reported human cases, 105 were fatal. Neutralising antibodies were found in two species of flying foxes (Johara *et al.*, 2001). Virus was sunsequently isolated from flying foxes (Chua *et al.*, 2002).

So what factors have triggered the clustered emergence of Hendra virus and ABLV? The hypothesis that both Hendra virus and ABLV are primarily maintained in *P. scapulatus* populations, and that change in the population dynamics of this species due to ecological changes has precipitated emergence is attractive. Having argued the former in Section 7.2.3 and 7.3.1 respectively, it now remains to explain the emergence. Historically, *P. scapulatus* made larger nomadic movements over much of northern Australia. These movements were irregular, reflecting the flowering patterns of preferred eucalypt species, which in turn reflect major climatic patterns. In contrast, the other species are more coastal in their distribution, and tend to be less nomadic. Permanent or semi-permanent urban populations of several hundred or several thousand are common in many Queensland coastal towns. The presence of *P. scapulatus* in these urban camps was limited and infrequent. This, however, appears to have changed over the last decade, with *P. scapulatus* increasingly reported in these camps in large numbers. This phenomenon is attributed to changed foraging movements related to the reduction of traditional food resources as a consequence of landuse change

and climatic change. Thus, according to the hypothesis, the increased contact with urban flying fox populations has resulted in an increased incidence or frequency of Hendra virus and ABLV infection in these populations, and thus an increased probability of human/livestock exposure.

7.5 Further research recommendations

The research presented in this thesis has substantially advanced understanding of the origins of Hendra virus and ABLV in Australia, the ecology of infection of both agents in Australian bat, and risk factors for infection for both in bat and non-bat species. In addition, modelling has examined the dynamics of Hendra virus infection in a flying fox population, and hypotheses have been generated for the maintenance of both HeV and ABLV, and for their clustered emergence. However, further research is needed in several areas, particularly in relation to Hendra virus. Firstly, further observational and experimental studies are necessary to establish the route of HeV excretion and the mode of transmission in flying foxes. The inclusion of *P. scapulatus* in experimental studies would enable investigation of species-related host competency. Observational studies should encompass longitudinal studies of free-living populations to investigate temporal patterns of infection. Secondly, the roles of vertical transmission and latency in the transmission and maintenance of HeV in flying foxes need to be clarified through observational, experimental and modelling studies. Thirdly, more sophisticated modelling is needed to determine a model that best fits the observed dynamics of Hendra virus infection in flying foxes. This approach should consider heterogenous mixing and non-linear transmission. In relation to ABLV, further research is needed to understand the mechanism of maintenance of ABLV infection in flying fox and microbat populations. Methodologies used in the study of lyssaviruses in terrestrial and bat species elsewhere could be applied to ABLV. In addition, ABLV isolates from geographically discrete bat populations would facilitate phylogenetic studies and an understanding of the evolution of ABLV.

This project has provided a rare opportunity to investigate the emergence and ecology of two novel viruses in a global environment of increasing infectious disease emergence and increasing emergence from wildlife reservoirs. The emergence of both has had significant public and animal health impacts, and has fostered a heightened awareness of emerging infections disease in Australia and internationally. As the remaining questions are answered, our understanding of the ecology of Hendra virus and ABLV will become more

complete, and a fascinating chapter in animal and public health in Australia can be closed.

Appendix 1 Bat handling protocols

Preamble

The following guidelines address several field scenarios. They have been developed by the DPI&F bat research group to facilitate the safe conduct of their research. Emphasis is on handler safety and humane treatment of animals.

Captive flying foxes

1. Personnel
   - Two experienced handlers, or a trainee handler under direct supervision of at least one experienced handler
   - All personnel should have current rabies and tetanus vaccination.
   - All personnel should have read and understood these guidelines as part of an induction process.
   - Personnel should receive procedural directions from the leader prior to commencing each activity.

2. Protective clothing
   - Long sleeved overalls.
   - Boots
   - Glasses or face shield
   - Double-glove with N-DEX® puncture resistant gloves or N-DEX® under leather 'riggers' gloves, leather 'welders' gloves, or chain mesh gloves.

3. Catching, restraint and examination
   - Penned flying foxes should be approached quietly, and caught from behind as they move away from the handler on the roof or side of the cage.
   - The handler must ensure firm restraint of the flying fox's head before attempting to unhook itsfeet and thumbs from the wire.
   - The animal's head, neck and shoulders should be grasped with one hand, while the other hand gathers the wings to the body. Head and wing control are the key to safe handling.
   - If necessary, a (gloved) assistant can help unhook claws once the flying fox is effectively held.

- The flying fox can then be firmly wrapped in a towel (wings folded alongside its body and its head covered) and held in dorsal recumbency on a table if possible. Alternatively, a purpose-made bat restraint board (which allows hands-free restraint) can be used. Chemical restraint using an inhalation or injectable agent can also be used (see below).
- Leather gloves can be removed to examine or bleed the flying foxl. Particular care should be taken at this stage to ensure the head is securely restrained. The abdomen, leg, wing, etc can then be exteriorised for examination or bleeding.
- N-DEX® puncture resistant gloves should be worn throughout the whole procedure to minimise exposure to blood and other body fluids.
- Used needles, syringes and swabs should be properly disposed of into an approved 'sharps' container.
- When examination or bleeding is completed, the handler replaces his/her leather gloves, and after ensuring firm restraint of the head, picks up the flying fox in the towel and releases it onto the wire roof or side of the pen.
- Used equipment and protective clothing should be thoroughly cleaned on site in Virkon™ or sealed in an autoclave bag and transported to the lab.
- Blood samples or animals should be transported to the lab according to approved protocols.

4. First-aid
    - A well-stocked field first-aid kit should be immediately accessible.
    - A bucket of Virkon™, Hibicleans™, or other appropriate antiseptic should be immediately accessible.
    - Any scratches or bites, or any blood, urine or faeces contamination of handlers should be immediately washed thoroughly and informed medical advice obtained.
    - Animals that inflict a bite should be identified to enable possible euthanasia and diagnostic testing.

Wild-caught flying foxes

1. Personnel
    - As for captive flying foxes. A minimum of three people is preferable.

2. Protective Clothing
    - As for captive flying foxes

3. Catching, Restraint and Examination Procedures
    - o As for captive flying foxes.

    In addition..
    - o Recognised capture techniques should be used. These include mist-netting, harp-trapping, or (for *P. scapulatus*) camp ambush and hand capture.
    - o Wild-caught flying foxes should be placed in individual in calico holding bags.
    - o Wild-caught flying foxes should preferably examined under chemical restraint using an inhalation anaesthetic or an injectable agent. For the former, the animal's head is exteriorised from the bag, the anaesthetic mask placed firmly over its face, and the animal induced. For the latter, the animal's leg or wing is exteriorised and the injection given. Physical restraint is as described for captive flying foxes.

4. First-aid
    - o As for captive flying foxes
    - o Animals that inflict a bite should be euthanased and appropriate diagnostic tests performed.

Wild-caught microbats

1. Personnel
    - o As for wild-caught flying foxes

2. Protective Clothing
    - o As for wild-caught flying foxes
    - o For small microbats, double or triple-gloving with Ndex gloves alone is adequate.

3. Catching, Restraint and Examination Procedures
    - o The general principals described for wild-caught flying foxes apply.

    In addition..
    - o Microbats should be captured using recognised techniques. These include mist-netting, harp-trapping, scoop-netting and manual removal from buildings, mines, tunnels.
    - o The bat should be restrained by grasping it's head and body or scruff with the fingers of one hand.

- The handler should ensure effective restraint of the head or scruff before attempting to unhook feet and thumbs.
- Captured bats should be placed individually or collectively (depending on species) into calico holding bags.
- Bats can then be individually removed from the bag for processing. Particular care is taken at this stage to ensure the animal's head or scruff is securely restrained. The animal's abdomen, leg, wing etc can then be exteriorised for examination or bleeding.

4. First-aid
   - As for wild-caught flying foxes

Appendix 2 Neutralizing antibody titres to Hendra virus in 68 flying foxes non-randomly (convenience) sampled from a captive colony in May 1996.

Table A2.1: Neutralizing antibody titres to Hendra virus in 68 flying foxes non-randomly (convenience) sampled from a captive colony in May 1996.

Accession number	Sample number	Species	Sex	Age class	HeV SNT titre
139432	59	*Pteropus alecto*	Female	Immature	<5
139432	40	*Pteropus poliocephalus*	Female	Immature	<5
141713	127	*Pteropus poliocephalus*	Female	Immature	<5
141713	128	*Pteropus poliocephalus*	Female	Immature	<5
141713	129	*Pteropus poliocephalus*	Female	Immature	<5
141713	130	*Pteropus poliocephalus*	Female	Immature	<5
141713	131	*Pteropus poliocephalus*	Female	Immature	<5
141686	155	*Pteropus poliocephalus*	Female	Immature	<5
141686	160	*Pteropus poliocephalus*	Female	Immature	<5
141713	145	*Pteropus poliocephalus*	Male	Immature	<5
141686	157	*Pteropus poliocephalus*	Male	Immature	<5
141686	150	*Pteropus poliocephalus*	Female	Mature	<5
141686	162	*Pteropus poliocephalus*	Female	Mature	<5
141686	163	*Pteropus poliocephalus*	Female	Mature	<5
141686	166	*Pteropus poliocephalus*	Female	Mature	<5
141686	169	*Pteropus poliocephalus*	Female	Mature	<5
141686	156	*Pteropus poliocephalus*	Male	Mature	<5
141686	158	*Pteropus poliocephalus*	Male	Mature	<5
141686	164	*Pteropus poliocephalus*	Male	Mature	<5
141686	165	*Pteropus poliocephalus*	Male	Mature	<5
141686	167	*Pteropus poliocephalus*	Male	Mature	<5
141686	168	*Pteropus poliocephalus*	Male	Mature	<5
139432	63	*Pteropus alecto*	Female	Unknown	<5
141713	119	*Pteropus alecto*	Female	Unknown	<5
141713	123	*Pteropus alecto*	Female	Unknown	<5
141713	124	*Pteropus alecto*	Female	Unknown	<5
141713	125	*Pteropus alecto*	Female	Unknown	<5
141713	126	*Pteropus alecto*	Female	Unknown	<5
139432	35	*Pteropus poliocephalus*	Female	Unknown	<5
139432	36	*Pteropus poliocephalus*	Female	Unknown	<5
139432	37	*Pteropus poliocephalus*	Female	Unknown	<5
139432	38	*Pteropus poliocephalus*	Female	Unknown	<5
139432	51	*Pteropus poliocephalus*	Female	Unknown	<5
139432	52	*Pteropus poliocephalus*	Female	Unknown	<5

Table A2.1 continued next page

Table A2.1 continued

139432	53	*Pteropus poliocephalus*	Female	Unknown	<5
139432	56	*Pteropus poliocephalus*	Female	Unknown	<5
139432	57	*Pteropus poliocephalus*	Female	Unknown	<5
141713	138	*Pteropus poliocephalus*	Female	Unknown	<5
141713	140	*Pteropus poliocephalus*	Female	Unknown	<5
141713	141	*Pteropus poliocephalus*	Female	Unknown	<5
141713	142	*Pteropus poliocephalus*	Female	Unknown	<5
141713	143	*Pteropus poliocephalus*	Female	Unknown	<5
141713	148	*Pteropus poliocephalus*	Female	Unknown	<5
141686	159	*Pteropus poliocephalus*	Female	Unknown	<5
141686	161	*Pteropus poliocephalus*	Female	Unknown	<5
139432	58	*Pteropus alecto*	Male	Unknown	<5
139432	61	*Pteropus alecto*	Male	Unknown	<5
139432	34	*Pteropus poliocephalus*	Male	Unknown	<5
139432	39	*Pteropus poliocephalus*	Male	Unknown	<5
139432	41	*Pteropus poliocephalus*	Male	Unknown	<5
139432	42	*Pteropus poliocephalus*	Male	Unknown	<5
141713	132	*Pteropus poliocephalus*	Male	Unknown	<5
141713	133	*Pteropus poliocephalus*	Male	Unknown	<5
141713	134	*Pteropus poliocephalus*	Male	Unknown	<5
141713	137	*Pteropus poliocephalus*	Male	Unknown	<5
139432	60	*Pteropus alecto*	Male	Immature	20
141713	144	*Pteropus poliocephalus*	Male	Immature	5
141713	146	*Pteropus poliocephalus*	Male	Immature	10
141713	147	*Pteropus poliocephalus*	Male	Immature	10
141686	154	*Pteropus poliocephalus*	Male	Immature	10
141686	151	*Pteropus poliocephalus*	Female	Mature	160
139432	64	*Pteropus alecto*	Female	Unknown	40
141713	118	*Pteropus alecto*	Female	Unknown	10
141713	120	*Pteropus alecto*	Female	Unknown	10
139432	50	*Pteropus poliocephalus*	Female	Unknown	40
139432	55	*Pteropus poliocephalus*	Female	Unknown	10
141713	139	*Pteropus poliocephalus*	Female	Unknown	20
139432	43	*Pteropus poliocephalus*	Male	Unknown	40

Appendix 3 Characteristics of 17 flying foxes non-randomly (convenience) sampled from a captive colony and screened for Hendra virus between August and November 1996.

Table A3.1: Characteristics of 17 flying foxes non-randomly (convenience) sampled from a captive colony and screened for Hendra virus between August and November 1996.

Lab Number	Sex	Microchip number	Age	HeV serostatus
BR 150	Female		Mature	neg
BR 151	Female	0001DAD405	Mature	pos
BR 152	Male	0001DB05DC	Mature	pos
BR 153	Female		Mature	neg
BR 155	Female		Immature	neg
BR 156	Male		Mature	neg
BR 158	Male	0001DB0BDE	Immature	neg
BR 159	Female		Unknown	neg
BR 160	Female	0001DB8BE0	Mature	neg
BR 161	Female		Unknown	neg
BR 162	Female		Mature	neg
BR 163	Female	0001DB8B51	Mature	neg
BR 164	Male		Mature	neg
BR 165	Male		Mature	neg
BR 166	Female		Mature	neg
BR 169	Female	0001D302AE	Mature	neg
BR 111	Female		Mature	neg

Appendix 4 Excel™ spreadsheet models of Hendra virus infection dynamics in a flying fox population

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