Setting up camp in Adelaide:
Ecological Insights into the Range Expanding Grey-headed Flying Fox (*Pteropus poliocephalus*).

by

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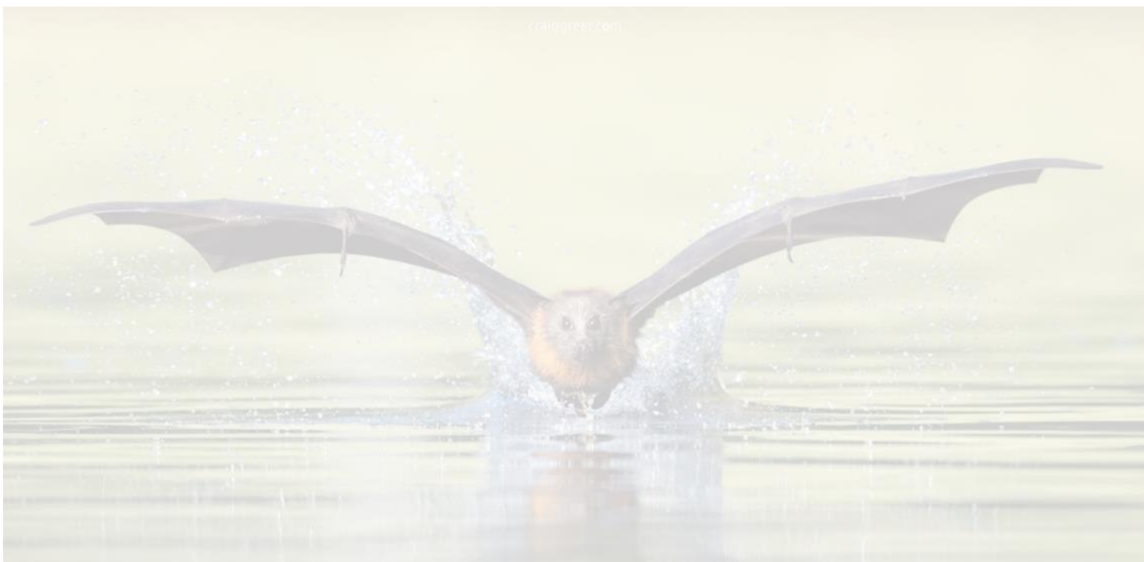

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Abstract:

Grey-headed flying foxes, *Pteropus poliocephalus*, classified as Vulnerable under the EPBC Act, 1999, historically ranged from Melbourne in Victoria to Ingham in Queensland. However, for the first time, a population set up camp close to the Adelaide city centre in 2011, expanding the range of the species. I wanted to investigate what had motivated them to move to Adelaide including whether there are 'pull' factors at work; environmental factors attracting flying foxes into the area. Specifically, I wanted to investigate how they were utilising the greater Adelaide landscape to forage and what they were eating. Furthermore, I wanted to measure the population's exposure to viruses of zoonotic potential to investigate whether their arrival poses a risk to public health.

Using GPS telemetry, I tracked individual flying foxes (n=5) to document their Spring foraging movements and activities. Despite being a highly mobile species, the mean core foraging range estimate was only 7.3 km^2 and maximum foraging distance from the camp in the Botanic Park was 9.5 km with most foraging occurring within a 4 km radius which indicates they found sufficient foraging resources entirely within the residential area of Adelaide on streets, parks and residences. They foraged on introduced tree species either not native to South Australia or exotic to Australia. Movements of individuals also indicated persistent utilisation of same foraging sites over many days, regular use of water resources and the use of air space around Adelaide International airport.

To further document the diet of Adelaide's flying foxes on a larger scale, I used DNA metabarcoding on 161 faecal bat samples to confirm which plants they were eating. I found that their diet included 40 operational taxonomic units (OTU) across 15 orders, 15 families and 10 genera. Over 86% of all plant sequences belonged to just three orders: Rosales (54.35%; including the families Rosaceae and Moraceae, the latter incorporating the genus *Ficus*), Myrtales (21.63%; including the family Myrtaceae which includes the genera *Eucalyptus, Angophora* and *Corymbia*) and Malpighiales

(10.26%; including the family Salicaceae which incorporates the genus *Populus*). The genus, *Ficus*, from the *Moraceae* family and Rosales order, which is not native to South Australia accounted for 22.10% of the sequences identified while the family, *Myrtaceae* which contains the genus *Eucalyptus* and is native to Australia accounted for 21.56% of all sequences. Overall, the majority (75%) of sequences were aligned to plant OTUs that could be considered either non-native to South Australia or exotic to Australia which indicates that Grey-headed flying foxes have been able to establish a camp in Adelaide as a result of the human-modified landscape.

On the global scale, flying foxes are known to act as spill-over hosts for emerging infectious pathogens including viruses of zoonotic potential. I therefore sampled a total of 301 flying foxes in the camp, over six serosurveys and investigated, using a multiplex Luminex binding assay, the levels of exposure to known viruses of zoonotic significance. I found strong serological evidence of common exposure to Cedar (apparent seroprevalence; AP = 26.6%; 95%CI: 21.7%-31.9%), Ebola Zaire (AP = 18.9%; 95%CI:14.7%-23.8%), Hendra (AP = 43.2%; 95%CI: 37.5%-49%), Severe acute respiratory syndrome (AP = 31.6%; 95%CI: 26.4%-37.1%), and Tioman (AP = 95.7 %; 95%CI: 92.7%-97.7%) virus antigens. Temporal variation in antibody levels suggests that antibodies to Hendra virus and Tioman virus may wax and wane on a seasonal basis. For all viruses, exposure could have occurred anywhere along the flying fox species distribution range continuum. However, I found no serological evidence of exposure to Middle Eastern respiratory syndrome virus (AP = 0.7%; 95%CI: 0.01%-2.4%) or Australian bat lyssavirus (AP = 0.0%; 95%CI: 0.0%-1.22%), despite a case caused by the latter virus being diagnosed in a bat from the camp in 2012.

In conclusion, I found the flying foxes have been attracted to Adelaide because of the abundance of foraging resources which has allowed them to thrive, and the population has been exposed to many viruses of zoonotic potential which may pose a biohazard risk to the human inhabitants of Adelaide.

Declaration:

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will in the future be used in submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

I acknowledge that copyright of published works contained within this thesis resides with the copyright holder of those works.

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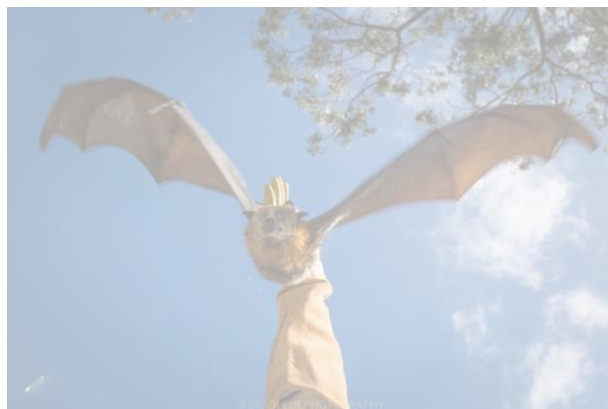

Thesis structure:

This thesis is presented as a 'Thesis by Publication' and includes a combination of accepted, submitted under review and finalised manuscripts yet to be submitted. Each chapter either forms a separate scientific manuscript or is the combination of two scientific manuscripts (some content cannot be published yet because of political sensitivities). Accordingly, some repetition between chapters exists and/or in the introduction and methods sections. For consistency I have standardised chapter formatting throughout this thesis and referenced other chapters where appropriate to avoid excessive duplication. For details of the journal and stage of each manuscript in the publication process please refer to 'Statement of Authorship' accompanying each chapter. All references to manuscripts/chapters of this thesis reflect the current status of that manuscript in the publication process (published, in press or in preparation). Included are citation details of manuscripts published or submitted by collaborators as a consequence of this research, for which I am a co-author.

List of included publications arising from this research:

1. **Boardman WSJ,** Roshier D, Reardon T, Burbidge K, McKeown A, Westcott DA, Caraguel CGB, and Prowse TAA. (2021, [Chapter 2]). Spring foraging movements of an urban population of Grey-headed flying foxes (*Pteropus poliocephalus)*. *Journal of Urban Ecology*, 1-10. doi: 10.1093/jue/juaa034

2. **Boardman WSJ,** Baker ML, Boyd V, Crameri G, Peck GR, Reardon T, Smith IG, Caraguel CGB, and Prowse TAA. (2020, [part of Chapter 4]). Serological evidence of exposure to Severe Acute Respiratory Syndrome coronavirus (SARS-CoV-1) in the Grey-headed flying fox (*Pteropus poliocephalus*). *Transboundary and Emerging Diseases*, 00:1–5. doi: 10.1111/tbed.13908

3. **Boardman WSJ,** Baker ML, Boyd V, Crameri G, Peck GR, Reardon T, Smith IG, Caraguel CGB, Prowse TAA. (2020, [Chapter 5]). Seroprevalence of three paramyxoviruses; Hendra virus, Tioman virus, Cedar virus and a rhabdovirus, Australian bat lyssavirus, in a range expanding fruit bat, the Grey-headed flying fox (*Pteropus poliocephalus*). *PLOS One*, doi: 10.1371/journal.pone.0232339

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1. **Boardman WSJ,** Bradshaw C, Prowse TAA, Crameri G, Westcott DA and Caraguel CGB. (2015). What are fruit bats doing in my backyard? Researching the disease and ecological implications of Grey-headed flying foxes in South Australia. *Proceedings of the 6th International Conference of the Wildlife Disease Association*, Sunshine Coast, Queensland, Australia, August 2015

2. **Boardman WSJ,** Burbidge K, Bradshaw C, Prowse TAA, Crameri G, McKeown A, Westcott DA, Reardon T and Caraguel CGB. (2016). What are flying foxes doing in Adelaide? Researching the disease and ecological implications of Grey-headed flying foxes in South Australia. *Proceedings of the South Australia Natural Resource Management Conference*, Adelaide, South Australia, April 2016

3. **Boardman WSJ,** Bradshaw C, Prowse TAA, Crameri G, McKeown A, Westcott DA, Reardon T and Caraguel CGB. (2016). What are fruit bats doing in Adelaide? Researching the disease and ecological implications of Grey-headed flying foxes in South Australia. *Proceedings of the Australasian Bat Society Conference*, Hobart, Tasmania, April 2016.

4. **Boardman WSJ,** Burbidge K, Bradshaw C, Prowse TAA, Crameri G, McKeown A, Westcott DA, Reardon T and Caraguel CGB. (2016). Spatial movements of Grey-headed flying foxes *Pteropus poliocephalus* in Adelaide and their foraging in Spring. *Proceedings of the Australasian Bat Society Conference*, Hobart, Tasmania, April 2016.

5. **Boardman WSJ,** Caraguel CGB, Baker ML, Boyd V, Peck G, Smith IG, Prowse TAA. (2018). Don't bat an eyelid – flying foxes, emerging pathogens and people: Eco-epidemiological measurements and models of bat-vectored diseases. *Workshop for the SA Health Zoonoses Working Group*, Adelaide, South Australia, August 2018.

6. **Boardman WSJ,** Caraguel CGB, Baker ML, Boyd V, Peck G, Smith IG, Prowse TAA. (2018). Seroprevalence of flying fox vectored viruses in the Adelaide Camp of Grey-headed flying Foxes: An update. *Workshop for the South Australia Wildlife Health Network*, Adelaide, South Australia, October 2018

7. **Boardman WSJ,** Caraguel CGB, Baker ML, Boyd V, Peck G, Smith IG, Prowse TAA. (2019). On a Wing and a Prayer: Investigating the viral seroprevalence of a range expanding species of flying fox the Grey-headed flying fox (*Pteropus poliocephalus*). *Proceedings of the European Association of Zoo and Wildlife Veterinarians Conference*, Kolmarden, Sweden, July 2019.

Other publications and submissions associated with this research:

1. Schaer J, **Boardman WSJ**, McKeown A, Westcott DA, Mastuschewski K, Power ML. (2019). Molecular investigation of Hepatocystis parasites in the Australian flying fox *Pteropus poliocephalus* across its distribution range. *Infection, Genetics and Evolution,* doi: 10.1016/j.meegid.2019.103978

2. McDougall FK, **Boardman WSJ**, Gillings M, Power ML. (2019). Bats as reservoirs of antibiotic resistance determinants: A survey of class 1 integrons in Grey-headed Flying Foxes (*Pteropus poliocephalus*). *Infection, Genetics and Evolution*, 70: 107-113. doi: 10.1016/j.meegid.2019.02.022

3. Sánchez C, Reardon T, O'Leary M, Altizer S, **Boardman WSJ**. (2019). Body condition predicts Grey-headed flying fox (*Pteropus poliocephalus*) foraging movements in an urban landscape. *Movement Ecology,* Submitted 2020.

4. McDougall FK, **Boardman WSJ,** Power ML. Genetic characterisation of beta-lactam resistant *Escherichia coli* from Australian fruit bats: Detection of resistance to critically important antimicrobials and extraintestinal pathogenic traits. *Microbial Genomics,* Submitted 2020.

5. McDougall FK, Wyres KL, Judd LM, **Boardman WSJ,** Holt KE, and Power ML. Novel strains of *Klebsiella africana* and *Klebsiella pneumoniae* in Australian Fruit Bats (*Pteropus poliocephalus*). *Proceedings of the Royal Society B: Biological Sciences,* Submitted 2021.

Chapter 1: Introduction

1.1 Range Expansion of Wildlife

1.1.1 Natural species range expansion

The study of the geographical range limits of species and the reasons why species occur where they do, are essential themes in ecology (Cayuela et al, 2018). Each species' distribution range is dynamic, fluctuates over time and is affected by an individual's ability to disperse from its site of birth to into a new habitat or area to survive and breed (Baguette & Van Dyck, 2007; Cayuela et al, 2018). Dispersal is an important process that expedites population and range expansion by providing a mechanism for colonisation or metapopulation dynamics (Walton et al, 2018). It is defined as a directed movement away from a previously-used area and into a new area that occurs irregularly (usually once during an individual's lifetime), where both the source and destination areas are usually used for an extended period (Teitelbaum and Mueller, 2019) . Dispersal can be further categorised as passive or active. In passive dispersers, movement is mainly driven by extrinsic factors such as animals, wind or ocean currents (Burgess, et al, 2016; Cayuela et al, 2018). In active dispersers, dispersal often infers specialised large-scale movements of animals away from their original location (Cote et al, 2017; Cayuela et al, 2018). Dispersal is distinguished from migration, which is a periodic, 'two-way, out and back movement', and from foraging movements which are defined as 'frequent, short-distance movements to locate food resources' (Cote et al, 2017).

A further distinct type of animal movement is nomadism where the movements of individuals or populations of animals move frequently between locations with irregular timing and/or direction, producing both within-year and between-year variability in location and movement patterns. Nomadic movements often produce large range sizes (Teitelbaum and Mueller, 2019). Nomadism

usually occurs in highly variable, resource-limited environments and can provide a mechanism of escape from natural disasters, predators and parasites.

Range expansion, which can be considered a natural phenomenon driven by each species' need to find a resource or ecological niche that best suits their evolution and population growth, is an essential component of evolution (Walton et al, 2018). When animals have dispersed and expanded their range successfully by finding and occupying new environments that are suitable for supporting their biological needs, this is defined as colonisation.

Three patterns of dispersal (Krebs, 2009) and thus range expansion, occur, and include: (i) *diffusion*, which is the gradual movement of a population over hospitable terrain over several generations; (ii) *jump dispersal*, which is the movement of individuals across large distances followed by successful formation of a population in a new area, and occurs in a short time relative to the longevity of the species and often over unsuitable terrain; and (iii) *secular dispersal*, which is where diffusion occurs over evolutionary time-scales and a species undergoes extensive evolutionary change as it gradually adapts to a new environment.

When species shift their range, they can encounter a collection of new selection pressures (Suarez and Tsutsui, 2008), e.g., temperature and humidity extremes, seasonal food availability, altitude; to which they must adapt or perish. In response to these selection pressures, evolution can affect the population dynamics of range extension which can then initiate further feedback on the evolutionary processes (Burton and Travis, 2008).

1.1.2 Anthropogenic causes of species range expansion

Anthropogenic causes of species range expansion can occur due to the: (i) intentional introduction of an exotic species; (ii) human-induced alteration of ecosystems (Ancilloto et al, 2016); (iii) human-

mediated biosecurity failure; (iv) rewilding, translocation and conservation corridors; and (v) climate change. In recent years, global climate change is outpacing the potential for species to adapt (Bradshaw et al, 2006; VanDerWal et al, 2013) and has increasingly driven changes in species distributions (Walther et al, 2002; Root et al, 2003) which are often towards the pole and higher elevation (VanDerWal et al, 2013). However, increasingly, there are reports of east-west directional changes across longitudes and even expansions towards tropical latitudes and lower elevations (Lenoir and Svenning, 2014). Paradoxically, when considering the creation of conservation corridors to facilitate wildlife movement, diffusion range expansion can sometimes occur more quickly in low quality habitats than high quality habitats which suggests a compromise between faster movement in poor quality habitat and between an increased population growth in a high-quality habitat (Crone et al, 2019).

1.1.3 Synanthropy and Urbanisation

Synanthropy refers to undomesticated species (plant or animal) adapting to living closely alongside, and benefiting from, human beings. A commonly used and similar term is *urbanisation* which relates to the specific benefits gained by animals in cities. The benefit of living near humans for animals can relate to food resource or breeding site availability (McFarlane et al, 2012). Synanthropy is a strategy used by an assorted range of species. For example, in Australia some 30 species of bats, 22 species of the largest marsupial order, *Diprotodontia* (e.g., kangaroos, possums), several species each of rodents, bandicoots, introduced ungulates, introduced carnivores and lagomorphs make their homes in human-modified environments (McFarlane, 2015). Of these, 20 species of bats find worthwhile resources in urban environments and have been able to adapt (McFarlane, 2015). *Synanthropy* or *urbanisation* provides a net benefit to species by definition, but there are also negatives including injuries and fatalities (Richardson et al, 2020), and social disconnection associated with human disturbance. Proximity to humans can lead to human-wildlife conflict including issues related to noise and smell, physical attacks and fatalities (Richardson et al, 2020),

vehicle collisions (Soga and Gaston, 2020) and the increased possibility of zoonoses (McFarlane et al, 2012; Allen et al, 2017).

1.1.4 Flying foxes – a jump dispersal species

A jump dispersal event is often human-mediated and can involve invasive species such as the Mediterranean fruit fly (Karsten et al, 2013) and frogs (Everman and Klawinski 2013), but they can also occur naturally with documented examples for Red foxes (*Vulpes vulpes*) (Walton et al, 2018), many plants (Jordano 2016) and in Australia, flying foxes (Tidemann, 1999; van der Ree et al, 2006). In Australia, a change in the distribution range of the Black flying fox (*Pteropus alecto*) and the Grey-headed flying fox (*Pteropus poliocephalus*) has been observed in recent times. These species are volant and have the ability to cover large distances and complex topographies (Tidemann, 1999; van der Ree et al, 2006). The range of the Black flying fox has expanded in a southerly direction by approximately 10 km per year over the 75 years (Tidemann, 1999) and this has been suggested as a possible reason for the northern contraction of the Grey-headed flying fox. The range of the Grey-headed flying fox moved 336 km south between 1882 and the 1930's (Ratcliffe, 1931). Probably due to native vegetation clearing, Grey-headed flying foxes are now more reliant on vegetation in urban and peri-urban coastal areas (McFarlane, 2015). Now in its southernmost distribution, the Grey-headed flying fox has increased its range to include Melbourne (van der Ree et al, 2006), Bendigo, Canberra, Geelong and, in 2011, Adelaide (Westcott et al, 2011), which now represents the most westerly edge of the species' range (Fig. 3). The range expansion has been ascribed to several influences. These can be termed 'push-pull' factors (Byrne et al, 2019). Range expansion can be initiated by the 'pull' of sustainable resources and conditions in new areas or by the 'push' of diminished resources and declining conditions in their former range. Flying foxes might respond to 'pull' factors which may include enhanced food accessibility in new landscapes (McDonald-Madden et al, 2005) or 'push' factors which can include a worsening of resources in the former range of the species (Tidemann 1999; McDonald-Madden et al, 2005; van der Ree et al, 2006), competition with

other flying foxes (Webb and Tidemann 1995) or anthropogenic climate change (Parris and Hazell 2005).

1.2 Flying foxes in Australia

1.2.1 Bat taxonomy overview

Bats are a unique group of mammals within their own Order, Chiroptera, and are globally distributed, although individual bat species have their own geographical niche. There are 1,422 reported species of bats worldwide (Upham et al, 2020), making up approximately 20% of all mammal species (Jones et al, 2001). Conventionally, bats have been classified into two suborders based on their body size: the 'megabats', larger sized bats which are mostly frugivorous, and the 'microbats', smaller sized bats which are mostly insectivorous. However, recent phylogenetic studies revised this classification into the two sub-orders - Yinpterochiroptera and Yangochiroptera (Fig 1) (Teeling et al, 2005; Tsagkogeorga et al, 2015). The former consists of the horseshoe bat (*Rhinolophoidea*) and the old-world fruit bat (*Pteropodidae*) families which includes flying foxes, and the latter includes Bulldog bats (*Noctilionoidea)*, Vesper bats (*Vespertilionidae)* and Sac-winged or sheath-tailed bats (*Emballonuridae)*. About 80 bat species are reported across Australia and outlying islands including eight families, one of which is the *Pteropodidae* family.

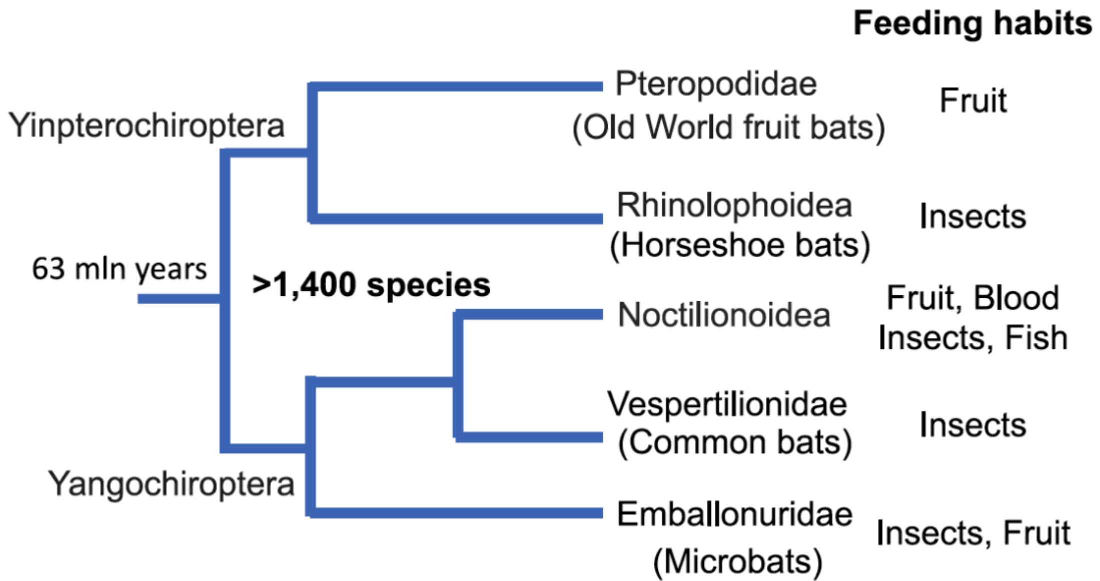

Fig 1. Major taxonomy and lineages of Order Chiroptera (Bats). Adapted from Gorbunova et al, 2020.

1.2.2 Bat species in the *Pteropus* genus.

Bats in the Family *Pteropodidae* are often called megabats or fruit bats. The family consists of a total of six subfamilies and 46 genera. *Pteropus* is a genus of megabats, which includes at least 60 extant species (Simmons 2005). They are often called flying foxes and are among the largest sized bats in the world. They are geographically distributed from the western Indian Ocean to the mid-Pacific islands in mostly tropical and sub-tropical climates (Fig.2). On many island systems they are the only endemic mammals and as such play a significant role in island ecology, primarily as seed-dispersers and pollinators (Fujita and Tuttle, 1991).

Fig 2. Geographical distribution of Pteropid bats (Adapted from Feldmann et al, 2002)

1.2.3 *Pteropus* species in Australia

There are four *Pteropus* species on mainland Australia and one on Christmas Island. The Christmas Island flying fox (*Pteropus melanotus natalis*) is classified as Critically Endangered under the Australian Environment Protection and Biodiversity Conservation (EPBC) Act, 1999 (Threatened Species Scientific Committee, 2001a) and its distribution is restricted to Christmas Island. The Spectacled flying fox (*Pteropus conspicillatus*) is classified as Endangered under the Australian EPBC Act (Threatened Species Scientific Committee, 2001b). The species is restricted to tropical rainforest areas between Ingham and Cooktown, between the Iron and McIlwraith Ranges of Cape York and is also found in Papua New Guinea and nearby islands, parts of Indonesia, and also the Solomon Islands (Duncan et al, 1999; Garnett et al, 1999). The Black flying fox (*Pteropus alecto)* is not listed under the Australian EPBC Act. The species is found around the northern coast of Australia (Western Australia, Northern Territory, Queensland and northern NSW) and inland wherever permanent water in rivers is found, as well as in Indonesia. The Little red flying fox (*Pteropus scapulatus*) is also not listed under the Australian EPBC Act. The species occurs from Shark Bay in WA through northern Australia, and down the east coast to northern Victoria, ranging far inland (the species has been

recorded in northern South Australia on two occasions) and is found in Papua New Guinea. The Grey-headed flying fox (*Pteropus poliocephalus*) is classified as Vulnerable under the Australian EPBC Act (Threatened Species Scientific Committee, 2001c) and "vulnerable to extinction" in the Action Plan for Australian Bats (Duncan et al, 1999). This species is Australia's only mainland endemic flying fox species and occurs in coastal areas from Ingham in northern Queensland to Adelaide in South Australia.

1.3 Bats as viral reservoir hosts

Extensive geographical distribution, in combination with high mobility, make bats ideal hosts and vectors for pathogen spread (Calisher et al, 2006). When this is coupled with new ecological pressures such as climate change and habitat loss, jump dispersal of populations of bats can contribute to pathogen translocation into new areas which is commonly termed 'pathogen pollution'. Bats have been widely implicated as reservoir hosts of many emerging and re-emerging viruses (Calisher et al, 2006; Luis et al, 2013; Ge et al, 2013). Molecular studies have confirmed that bats are putative reservoirs for numerous recently emerged zoonotic viruses, including Severe Acute Respiratory Syndrome coronavirus (SARS-CoV-1) (Ge et al, 2013), Ebola and Marburg haemorrhagic fever filovirus (Leroy et al, 2005; Towner et al, 2009) rabies and rabies-related lyssaviruses and many paramyxoviruses, including Nipah and Hendra viruses (Halpin et al, 2000; Chua et al, 2002; Drexler et al, 2012). Bats infected with these viruses often exhibit no clinical signs of disease (with the exception of lyssaviruses) (Wang et al, 2011) and in some cases appear to be persistently infected (Soyharti et al, 2011). Zoonotic viruses in more than 15 virus families have been identified in at least 200 species from 12 bat families around the world (Luis et al, 2013; Drexler et al, 2012; Wang et al, 2011). In a comparative analysis, Luis et al, (2013) showed that bats on average are more likely to be infected with more zoonotic viruses per species than rodents, adding weight to the idea that bats might be the primary source of emerging zoonotic viruses (Calisher et al, 2006).

1.3.1 Coronaviruses

Coronaviruses are significant emerging pathogens of humans and have caused outbreaks of Severe Acute Respiratory Syndrome (SARS) and Middle East Respiratory Syndrome (MERS) (de Groot et al, 2013; Banerjee et al, 2019), and most recently a new severe acute respiratory syndrome (known as COVID-19) associated with SARS-CoV-2 virus (Andersen et al, 2020). SARS associated with SARS-CoV-1 first emerged in late 2002 in Guangdong Province, southern China, spreading rapidly to Hong Kong and then to another 29 countries (Hu et al, 2017). Bats including several species of Chinese horseshoe bats (*Rhinolophus spp.*) from different locations in southern China were found to be infected with SARS-CoV-1 and are now considered the reservoir hosts (Guan et al, 2003; Hu et al, 2017). The seroprevalence to SARS-CoV-1 in some species of Chinese horseshoe bats was as high as 84.0% (Hu et al, 2017). However, no pathology has been reported in bats infected with SARS-CoV-1. MERS emerged in Saudi Arabia and then spread to other countries in Africa, Asia, North America and Europe. There has been a total of 2,500 cases and more than 850 deaths with an estimated case-fatality rate of 35.0% (Hu et al, 2017). The reservoir hosts of MERS are considered to be middle eastern insectivorous bats (Memish et al, 2013; Lau et al, 2018). In late 2019 – early 2020, a pandemic occurred associated with a new coronavirus, SARS-CoV-2, which most likely originated in (and recently spilled over from) insectivorous bats (Latinne et al, 2020) because it is part of a clade of closely-related SARS-related coronaviruses found in *Rhinolophus* spp. bats (Zhou at al, 2020). However, it is likely that a recombination event has occurred at some stage (Andersen et al, 2020). At the time of writing, there have been over 69 million reported human cases and over 1.573 million deaths in over 191 countries (Dong et al, 2020).

1.3.2 Filoviruses

Filoviruses have caused outbreaks of haemorrhagic disease in humans and nonhuman primates (Sanchez et al, 2001; Bempong et al, 2019) and there is a growing body of knowledge to suggest bats are the main reservoir hosts for these viruses (Chowell and Nishiura, 2014). Ebola virus disease first

appeared in 1976 with two concurrent outbreaks of acute viral haemorrhagic fever in humans in the Democratic Republic of Congo (DRC) (Laupland and Valiquette, 2014). Since these original cases, there have been many further outbreaks in east and west Africa (Bempong et al, 2019). Ebola virus has also been implicated as one of the most important causes of decline of African gorilla and chimpanzee populations in recent years (Vogel, 2003; Walsh et al, 2003; Walsh et al, 2005). Ebola virus is transmitted to humans through contact with bodily fluids and blood from another infected person or non-human primate, either by direct contact or indirectly from a contaminated environment (Walsh et al, 2005). Antibodies to Ebola viruses have been detected in three African fruit bat species (Leroy et al, 2005; Pourrut et al, 2009), in bats in Bangladesh (Olival et al, 2013), bats in Singapore (Laing et al, 2018) and in Chinese bat species (Yuan et al, 2012; Yang et al, 2017; Zhang et al, 2020). These studies provide a growing body of evidence that that infection with ebolaviruses or related filoviruses occur in bats not only from mainland Africa, but also Asia (Negredo et al, 2011; Olival and Hayman, 2014).

1.3.3 Paramyxoviruses

1.3.3.1 Hendra virus

Hendra virus, from the Paramyxovirus family is a member of the genus, *Henipavirus* which also includes Nipah virus. It was first described in 1994 (Murray et al, 1995) when it was identified as the cause of an outbreak of respiratory disease in horses (Edson et al, 2015). As of June 2020, over sixty outbreaks of Hendra virus have occurred in Australia (Queensland and NSW only), all involving infection of one or more horses (Business Queensland, 2021). To date, the case fatality rate is estimated at 75% for horses and 60% for humans (Field et al, 2011). Hendra virus events have mainly occurred in the cooler months in winter (Martin et al, 2016). No disease events have been documented in the subtropics during summer. In the northern tropics, events have been reported throughout the year. Serological evidence of infection with Hendra virus has been shown in all four species of pteropid bat that occur on mainland Australia (Breed et al, 2011; Edson et al, 2015). From

a non-probability serosurvey of 1,043 flying foxes across northern Australia, a crude seroprevalence of anti-Hendra virus antibodies was estimated to be 47% in 1996-1998 (Field et al, 2001). Serological evidence of Hendra virus infection has also been reported in six species of flying foxes from Papua New Guinea (Halpin et al, 1999). In 2009, Henipaviruses were detected in the African straw-coloured fruit bat (*Eidolon helvum*) in West Africa for the first time which indicates that endemicity extends beyond the Australasian region (Drexler et al, 2009). Black flying foxes and Spectacled flying foxes play the most active role in the transmission of Hendra virus to horses (Edson et al, 2015) and are considered the main source of infection for horses and humans (Edson et al, 2015). The virus is most frequently shed in the urine, but there is no evidence so far of viral shedding in the urine of Grey-headed flying foxes (Edson et al, 2015; Edson et al, 2019, Peel et al, 2019). In addition to urine spray and social grooming, direct contact with aborted foetuses and birthing fluids have been suggested as a route of virus shedding between flying foxes (Williamson et al, 1998). Despite only small amounts of virus being shed in urine, the use of pooled urine collected on tarpaulins under roost trees has proven effective for detection of the virus (Edson et al, 2015).

Cedar virus, a close relative of Hendra and Nipah viruses, was first identified in flying fox urine during Hendra virus screening in Queensland in 2009 (Marsh et al, 2012). However, no illness was observed in challenged animals (guinea pigs and ferrets) susceptible to other paramyxoviruses and thus its infectiousness and virulence are likely limited. Animals were able to mount an effective response with neutralising antibodies (Marsh et al, 2012).

1.3.3.2 Menangle Virus

Menangle virus, another Paramyxovirus, was isolated in 1997 from stillborn piglets at a large commercial piggery near Menangle in Australia (Philbey et al, 1998; Barr et al, 2012). Most sows carried their litters to term, but abortions occurred occasionally. Affected litters included mummified, autolysing and fresh stillborn piglets (Philbey et al, 1998; Barr et al, 2012). Two out of

250 humans in contact with the affected pigs had high antibody titres to the new virus, and both reported fever with a measles-like rash. Neither had direct exposure to flying foxes (Chant et al, 1998). A mixed camp of Grey-headed flying foxes and Little red flying foxes roosting seasonally within 200 m of the piggery carried neutralising antibodies (Philbey et al, 1998; Barr et al, 2012). However, other flying foxes in other camps, which were distant to and screened prior to the disease event at Menangle, also carried neutralising antibodies (Philbey et al, 1998; Barr et al, 2012). This suggests that the virus has been circulating in flying foxes for some time, but the virus needs a physically close intermediary species such as pigs for the spillover to occur. No known further cases have been reported.

1.3.3.3 Nipah Virus

Nipah virus was first isolated in 1999 from pigs and adult human males affected by fever and encephalitis during an outbreak in Malaysia and Singapore (Chua et al, 1999). Of 265 reported human cases, 105 were fatal (39.6 % case-fatality rate). Direct contact with infected pigs was identified as the main source of the human infection (Chua et al, 2000; Goh et al, 2000). Most of the humans affected in the Malaysian outbreak had a history of direct contact with live pigs, and most were adult male Chinese pig farmers (Chua et al, 1999). With suspicions that fruit bats were the most likely reservoir, Malaysian bats were targeted for surveillance. Consequently, the Large flying fox (*Pteropus vampyrus*) and the Small flying fox (*Pteropus hypomelanus*) were found to be the natural reservoir hosts for Nipah virus (Johara et al, 2001; Chua et al, 2002). Subsequently, it has been revealed that direct fruit bat-to-human and human-to-human transmission of Nipah virus has occurred in Bangladesh (Field et al, 2001; Yob et al, 2001; Gurley et al, 2007) and in India (Chatterjee 2018).

1.3.4 Lyssaviruses

1.3.4.1 Australian bat lyssavirus

The health investigation of a female Black flying fox in 1996 that was unable to fly, resulted in the isolation of a new lyssavirus species, Australian bat lyssavirus (ABLV) which is one of the 16 classified species of lyssaviruses within the family *Rhabdoviridae*. Surveillance subsequently confirmed the presence of ABLV from five different bat species, all four species of flying foxes in Australia (Field et al, 2004) and from an insectivorous bat, the Yellow-bellied sheath-tailed bat (*Saccolaimus flaviventris*), with two distinct lineages apparently circulating in insectivorous and frugivorous bats (Warrilow et al, 2002; Guyatt et al, 2003). Clinically affected bats exhibit neurological signs including limb paralysis or weakness, rapid involuntary eye movements, inability to swallow, hypersensitivity, biting, vocalisation and a hoarse cry (Field, 2005). Since 1995, a total of 342 bats have been confirmed to be infected in Australia with most cases coming from Queensland (63.2%) and NSW (24.3%) (Cox-Witton, 2020). Only one case has been reported from South Australia in 2012 in a Grey-headed flying fox (Cox-Witton, 2020). Recent research indicates evidence of exposure exists in six species of insectivorous bats in Australia (Prada et al, 2019). There have also been three reported human cases resulting from direct contact with bats (Warrilow et al, 2002; Francis et al, 2014), and two cases in horses (Annand and Reid, 2014).

1.4 Flying fox ecology with an emphasis on the Grey-headed flying fox (*Pteropus poliocephalus*)

1.4.1 Distribution

The size of the distribution range varies across the four Australian pteropodid species. The Little red flying fox having the largest range (3.6 million km^2), followed by the Black flying fox (1.6 million km^2), the Grey-headed flying fox (0.7 million km^2), and the Spectacled flying fox, with the smallest range (0.1 million km^2) (Webb and Tidemann, 1996). Since 2011, the range of the Grey-headed flying fox has extended into South Australia with a population established permanently in Adelaide (Westcott et al, 2011). This range expansion can also be described as a long-distance, jump dispersal as the species has moved large distances over a short time period and colonised new habitats.

The Grey-headed flying fox is Australia's only mainland, endemic flying fox. It is found along the coastal regions of eastern Australia, with a distribution extending from central Queensland (~21 °S) to Melbourne, Victoria (~38 °S) (Roberts et al, 2012) and now to Adelaide (~35 °S) (Westcott et al, 2011). Occasional individuals have been observed on Bass Strait islands (Tidemann, 1999) and mainland Tasmania. It can also be found infrequently west of the Great Dividing Range (Tidemann, 1999). However, only a small part of this range is used at any one time, as the species selectively forages wherever food is available. As a result, the occurrence and relative abundance within its distribution range can vary widely between seasons and between years (Eby & Lunney, 2002).

There is a degree of sympatry where different flying fox species can roost in mix-species camps (Garnett et al, 1999; Tidemann et al, 1999; Parsons et al, 2006; Welbergen, 2008) and there are instances where all four species of mainland Australian pteropodids can co-occur in the same camp e.g. Finch Hatton in Queensland (Parsons et al, 2010).

Fig 3: Distribution of Grey-headed flying foxes (*Pteropus poliocephalus*) (2019 data).

http://www.environment.gov.au/webgis-framework/apps/ffc-wide/ffc-wide.jsf. Accessed 1 December 2020.

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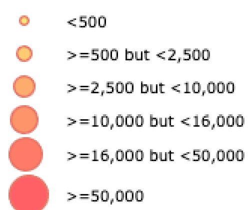

1.4.2 Roost habitat

Also known as ‘camps’, daytime roost sites of the Grey-headed flying fox often comprise thousands of individuals, and occur in rainforest, wetland, riparian or mangrove forests (Ratcliffe, 1931; Eby, 1991; Tidemann, 1999). The population of bats within a camp can vary substantially, often associated with changes in the availability and seasonality of flowers and fruits (Eby, 1991, Parry-Jones and Augee, 1992; Eby and Lunney, 2002). Large aggregations of bats can be linked to the flowering of nectar-rich species, particularly Eucalypts, over large areas (Parry-Jones and Augee, 1992, Eby, 1996). At night, this species will feed on blossoms and fruits away from their roosts and will use remnant forest patches of vegetation on cleared land and in urban areas (Eby, 1991; Tidemann, 1999). Roost sites are normally located near water, such as streams or rivers or near the coast (van der Ree et al, 2006). Camp vegetation includes rainforest patches, stands of *Melaleuca*, mangroves and riparian foliage (Nelson 1965; Ratcliffe 1931), but camps also use modified plantings (*Pinus* spp) in urban and suburban areas (van der Ree et al, 2006). The species will show some fidelity to roost sites with some roosts being used for decades (Ratcliffe, 1931; Tidemann, 1999), although new sites may form regularly as abundant food resources become available elsewhere.

1.4.3 Reproduction

Generally, female Grey-headed flying foxes do not reach full sexual maturity until three years of age (Hall, 2002). However, for some, pregnancy may occur in the second year (McIlwee and Martin, 2002). Mating occurs in autumn in the camp, after which time some larger camps can break up, reforming in late spring or early summer, as food resources become more abundant (Hall and Richards, 2000). Within a camp, males and females segregate in late September (L Collins 2020, pers. comm.) when females usually give birth after a six-month gestation, (Fig. 4), usually to single young, while twins are rare (L Collins 2020, pers. comm.). Lactation extends for four to five months or sometimes longer (L Collins 2020, pers. comm.) (Fig. 4). For four to five weeks after birth, the mother carries her single young when foraging (Tidemann, 1999). Once the young are completely furred,

they are left in maternal camps while their mothers forage and are then nursed by their mother after returning from foraging until they are independent at around 20 weeks (Hall & Richards, 2000). As adult female Grey-headed flying foxes conceive one young annually (Nelson, 1965), the population growth rate is slow relative to their size (Parry-Jones, 2001). This is further affected by the tendency of females to abort or abandon their young in response to food shortages (Tidemann, 1999). As a result, mass abortions and premature birth events are known to occur (L Collins 2020, pers. comm.).

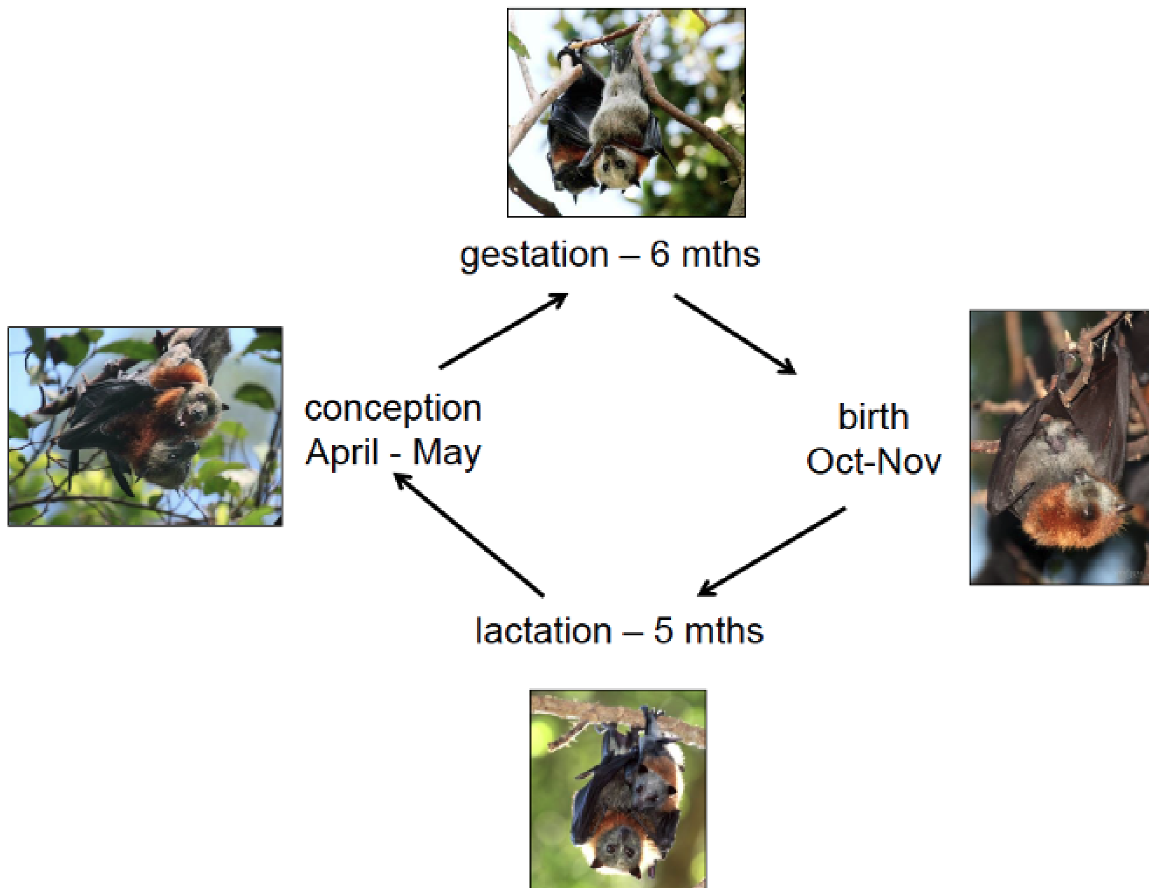

Fig 4: Reproductive cycle of the Grey-headed flying fox (Source: P.Eby, unpublished data)

1.4.4 Foraging & Diets & the role of flying foxes in the provision of ecosystem services

The Grey-headed flying fox has an important role in the health and maintenance of many ecosystems in eastern Australia. The species performs seed-dispersal and pollination ecosystem services for many native trees, including commercially important hardwood and rainforest species, such as native figs and palms (Tidemann, 1999; Shilton et al, 1999). The species contributes directly to the reproduction, regeneration and the evolution of forest environments. If the population size or range of Grey-headed flying foxes were to reduce further, then rainforest seed dispersal and pollination could be severely reduced (Richards, 2000).

The Grey-headed flying fox has a diverse native diet, which it supplements with introduced (Eby, 1996; Eby, 1998; Hall and Richards 2000; Parry-Jones and Augee, 1991) and cultivated plants (Hall and Richards 2000). It is a frugivore and nectarivore, which utilises many native vegetation communities including rainforests, open forests, closed and open woodlands, *Banksia* woodlands and *Melaleuca* swamps, rainforest fruits and nectar and pollen from the Eucalypt flowers (Genera: *Eucalyptus*, *Corymbia* and *Angophora*) (Duncan et al, 1999; Eby, 1996). Reliable resources during late gestation, birth and early lactation in winter and spring are required to avoid rapid weight loss in adults and poor reproductive success (Eby 1999; Parry-Jones and Augee 2001). Historically, in winter, resources for the Grey-headed flying fox were limited in distribution to a narrow coastal strip in Queensland and northern NSW (Eby, 1996). These coastal areas contained important winter and spring flowering species, particularly Broad-leaved Paper Bark (*Melaleuca quinquenervia*), Spotted Gum *(Corymbia maculata),* Swamp Mahogany (*Eucalyptus robusta*) and Forest Red Gum (*Eucalyptus tereticornis*) (Eby, 1996).

Few of these forests provide continuous foraging resources throughout the year, which can often result in the species moving in response to temporary food resources (Duncan et al, 1999; Eby 1996; Nelson 1965; Parry-Jones and Augee, 1992). Many Eucalypts have regular seasonal flowering

patterns (Eby 1996) but, depending on environmental conditions such as rainfall, some may not flower every year or have earlier or later flowering seasons. Flying foxes are not physiologically adapted to withstand local food shortages but must move in response to changes in the amount of and location of flowering events (Eby 1991; Eby and Lunney 2002).

1.4.5 Movement Patterns

Grey-headed flying foxes travel nightly to foraging areas, usually within 15 km of their camp (Tidemann, 1998), but they are capable of nomadic flights of up to 80 km from their camp to different foraging areas as food resources change (Field et al, 2016). With suitable winds, Grey-headed flying foxes can travel at speeds in excess of 35 km per hour for extended periods (Tidemann, 1998). Except in Spring and early Summer when pre-weaning juveniles stay, there is generally a complete exodus from the roost site at dusk (L Collins 2020, pers. comm.). During these times, juveniles fly in and out of the site throughout the night (Parry-Jones and Augee 1992). The Grey-headed flying fox is highly mobile and moves along the east and south-east coast of Australia in search of food (Tidemann 1998; Welbergen et al, 2020). Some individuals are estimated to move long distances (Tidemann and Nelson, 2004), moving nomadically among a network of roosts, up to 12,000 km annually in some cases although the mean distance travelled annually is 1554 km (Welbergen et al, 2020). While fidelity of individuals to certain branches within specific trees has been documented, for example during mating periods (Welbergen, 2005), individuals are otherwise thought to exhibit low fidelity to roosts locally, resulting in high estimated daily camp turnover rates (17.5 ± 1.3%) (Welbergen et al, 2020). This suggests that flying fox roosts form nodes in a vast continental network of dynamic “staging posts” through which mobile individuals travel far and wide (Welbergen et al, 2020).

1.5 Threats to Grey-headed flying foxes

1.5.1 Habitat loss, degradation and persecution

Grey-headed flying fox populations are thought to be declining in abundance. Ratcliffe (1931) suggested that as much as half the Australian flying fox population was likely to have been lost since European settlement. Fly-out surveys conducted between 1998-2001 suggested a decline from 566,000 to 400,000 animals, or approximately 30% (Threatened Species Scientific Committee, 2001c). As a consequence, this decline led to the species' being listed as Vulnerable under the EPBC Act (1999) under Criterion 1 – 'decline in numbers only' (Threatened Species Scientific Committee, 2001c). Habitat clearing and degradation are currently thought to be the main threats to the Grey-headed flying fox in particular and to flying foxes in general (Westcott et al, 2011). Since European settlement, significant habitat modification in the form of land clearing for both urbanisation and agriculture has occurred (Tidemann, 1999; Eby 2002) and Australia has lost approximately 38% of its native forests (Bradshaw et al, 2012). Habitat loss has resulted in a decrease in the variety of flowering and fruiting tree species, particularly those that usually have a high nectar output (Birt, 2000). *Melaleuca* forests once provided an important food source for flying foxes, but over 70% have been cleared since European settlement (Bradshaw et al, 2012). In addition, large areas containing Forest Red Gum (*Eucalyptus tereticornis*) and Spotted Gum (*Corymbia maculata*) have been cleared, both of which are important winter-flowering tree species (Birt 2000). Habitat clearance and degradation has also impacted flying fox roosting sites (Lunney and Moon, 1997). Whilst the species' specific roosting requirements is not clearly understood, habitat loss has probably encouraged flying foxes to set up daytime roosts in suburban areas (Tait et al, 2014). On a physiological level, habitat loss causes an increase in the animal's energy expenditure, as individuals need to fly greater distances from campsites to feeding areas as well as between individual campsites.

It has been argued that, as a consequence of habitat degradation, Grey-headed flying foxes must rely more heavily on resources from non-native flora in urban settings (Williams et al, 2006; Schmelitschek et al, 2009) or from rural areas such as commercial fruit farms. This leads to more frequent interactions with growers which can result in reactionary culling to reduce crop losses (Teagle 2002) through electrocution, shooting, or poisoning (Vardon et al, 1995; Tidemann et al, 1997; Birt, 2000). Flying foxes cause crop losses by puncturing fruit with their teeth and claws, soiling fruit from the orchard and damaging trees by breaking limbs, particularly new growth carrying next season's fruiting buds (Ullio, 2002). The Grey-headed flying fox is capable of causing direct losses to horticulturalists in NSW and is the main species responsible for crop losses in this state (Ullio, 2002). Shooting flying foxes has been the most common method in attempting to protect fruit crops (Teagle, 2002). Permit systems in NSW and Victoria currently enable culling of flying foxes whereas Queensland ceased issuing permits for shooting in 2008. However, the number of flying foxes shot illegally is unknown. As a result, the impact on population size and demographic structure of the Grey-headed flying fox remains difficult to quantify. The impact is more substantial than direct deaths alone would indicate, for a large proportion of animals shot in orchards are pregnant and lactating females (Parry-Jones 1993; Tidemann et al, 1997). Juveniles who remain in maternity camps and are dependent on their lactating females are known to die of starvation when lactating females are killed (Nelson 1965).

Since the early days of European settlement, the Grey-headed flying fox has been persecuted not only because of its impact on orchards but also its impact on human amenity (Ratcliffe 1931; Tidemann et al, 1997). Impacts on amenity usually occur when camps are close to residential areas. When camps are large, typically >15,000 individuals, the accompanying noise, smell and faeces may be considered unpleasant for members of the public (Parry-Jones and Augee 1992). Negative media reports on their amenity impact are also exacerbated by the impression that flying foxes pose a high disease risk. Undoubtedly, bat-mediated disease does pose some risk, however, health authorities

indicate that the concern is generally out of proportion to the risk and that, for Hendra virus at least, it is not associated with camps (Edson et al, 2015; Edson et al, 2019). Though on occasions residential areas can often intrude on existing flying fox camps, Grey-headed flying foxes can readily form camps in urban and residential areas (Markus and Hall 2004; Williams et al, 2006). City camps tend to be located in habitat fragments or botanic parks, the small size of which constrains the camp footprint often leading to high densities of roosting individuals causing damage to vegetation (Tidemann, 1999).

1.5.2 Climate change, heat stress events and electrocution

Heat-related deaths in Australian flying foxes have been recognised many times since European settlement. In January 2004, a heatwave in north-east NSW, coincided with a field study of reproductive output in Grey-headed flying foxes, providing an opportunity to measure the relative impact of temperature on the species (Eby et al, 2004). During the study, the ambient temperature exceeded 45°C and an estimated 5,000–7,000 individuals died. There was a significant impact on dependent young less than 4 months old, which represented 33.6% of the pre-heat wave population but 94.3% of dead individuals, while the percentage of adult females with young reduced from 84.1% to 66.5% (Eby et al, 2004).

Heatwaves are now becoming hotter, longer and more frequent under anthropogenic climate change (Steffen et al, 2014), which poses an unprecedented threat to biodiversity in general and flying foxes in particular. Heat stress die-offs for flying foxes are likely to become more frequent and widespread in the future (Ratnayake et al 2019). Between 1994 and 2007, over 30,000 flying foxes (including at least 24,500 Grey-headed flying foxes) were killed during 19 heat-stress events (Welbergen, 2008). Climate models predict continued increases in the intensity, duration and frequency of such extremes heat events and with it the likelihood that mortality events in flying foxes will increase in frequency and extent (Welbergen, 2008; Cowan et al, 2014). The effects of

heat-stress events are compounded by the increased frequency of bushfires which results in the loss of food resources. During 2019–2020, unprecedented bushfires burnt an estimated 5.8 million hectares of forest within the range of the Grey-headed flying fox (Boer et al, 2020). In addition, another unquantified cause of death in flying foxes is electrocution on powerlines, particularly in urban areas where a disproportionately high number of lactating females are often killed (Duncan et al, 1999).

1.6 Flying foxes in South Australia and issues associated with their arrival

1.6.1 Historical perspective

There are few historical records of flying foxes in South Australia. A flying fox was caught in Gawler in April 1920, but the species was unknown. A Little red flying fox was found in Vivonne Bay on Kangaroo Island in 1946 and another unknown species of flying fox was caught in Bordertown, SA in February 1954. In 1954 a flying fox, (likely a Little red flying fox) was photographed on Henley Beach. In 1998, a small temporary camp of Grey-headed flying foxes was recorded in Mt Gambier and again in Southeast South Australia in 2003 and 2007.

1.6.2 Range expansion into South Australia

Approximately twenty Grey-headed flying foxes were observed in the Botanic Gardens, Adelaide, in March 2010 (J Van Weenen 2020, pers. comm.). On the 1st April 2010, c. 600 Grey-headed flying foxes were identified in a patch of vegetation consisting of pine, eucalyptus and deciduous trees close to the Naracoorte Hospital, Naracoorte, South Australia. On 3rd May 2010, c. 50 bats were seen in a Pencil pine (*Cupressus sempervirens*) in a garden off Fullarton Rd, Adelaide. By 20th May 2010, the population in this location was estimated to c. 1,270, but by 28th May 2010 the flying foxes had vacated the area (J Van Weenen 2020, pers. comm.). On January 11th 2011, c.30 were observed in a American Cotton Palm (*Washingtonia robusta*) in the Adelaide Botanic Gardens (J Van Weenen

2018, pers. comm.). This population grew to c. 150 individuals on 7th April 2011 (J Van Weenen 2018, pers. comm.). After encouragement to leave the Botanic Gardens (using noise and light), by 11th June 2011 c. 350 bats were located in Aleppo pine trees (*Pinus halepensis*) in Botanic Park, adjacent to the Adelaide Zoo entrance (J Van Weenen 2018, pers. comm.).

1.6.3 Permanent camp establishment and population growth.

By 28th June 2012, the Botanic Park population of flying foxes numbered c. 500 individuals, and by 7 August 2013 the population had grown to c. 1,079 (J Van Weenen 2018, pers. comm.). Since that time, the Adelaide population count has waxed and waned depending on immigration, emigration, reproduction and heat stress events (Fig.5). Immigration in winter and emigration in summer has also been reported (L Collins 2020, pers. comm.). For example, the population size between June-November 2018 was c. 27,000 individuals but had declined to c. 17,000 by December 2018 - January 2019 (L Collins 2020, pers. comm.). A small camp has recently been established near Millicent in south-east South Australia (J Van Weenen 2018, pers. comm.).

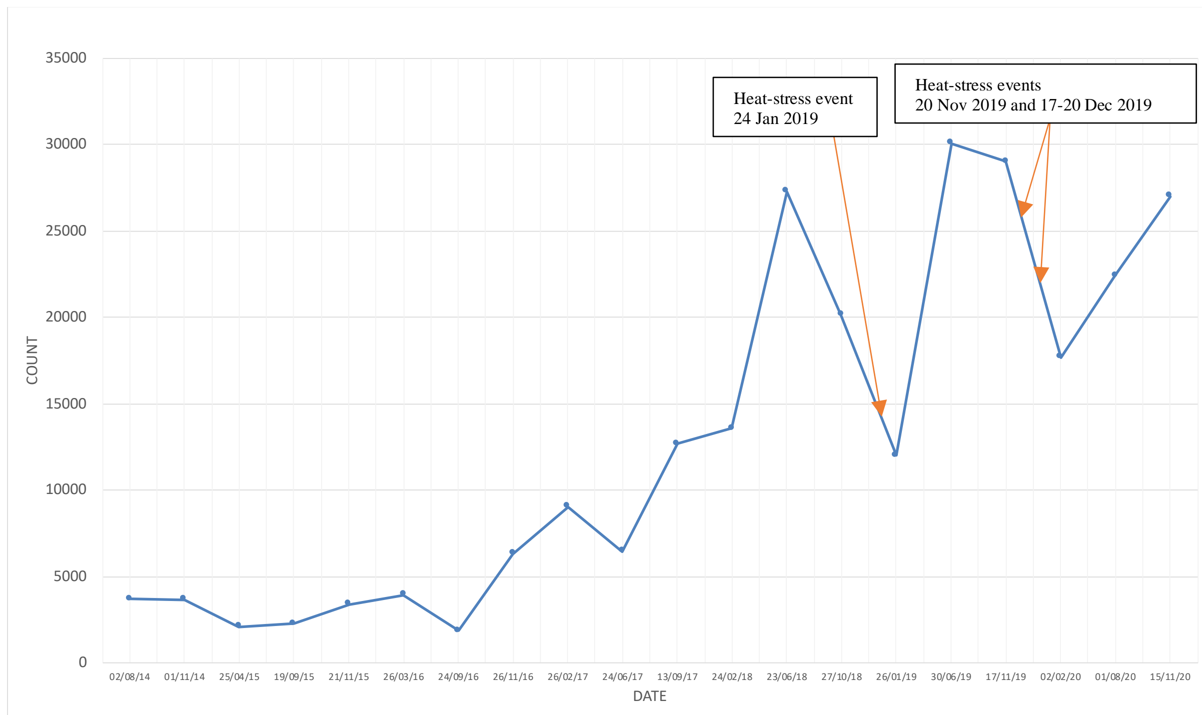

Fig 5: Time-series of Grey-headed flying fox counts in the Adelaide Botanic Park Camp over the 2014-2020 period with times of major recent heat-stress events (L Collins 2019, pers. comm.)

1.6.4 Threats to South Australian Grey-headed flying foxes

1.6.4.1 Heat-stress events

There have been several heat-stress events where the temperature in the camp is such that many animals die due to heat exhaustion (L Collins 2020, pers. comm.). Morbidity and mortality rates increase markedly when the camp temperature exceeds 42°C. (Welbergen et al, 2009). There have been 11 recorded heat-stress events in the Adelaide camp since 2013 (Table 1). Since January 2019, over two summer seasons, there have been three events with an estimated combined mortality of 13,500. On January 24th 2019, c. 4,000 bats died out of an estimated population of 20,000 when the temperature reached 46.6°C and between 17-20th December 2019, the temperature was >42°C for four consecutive days and c. 9,000 died out of a population of c. 25,000, with over c. 430 young bats taken into care. Since the summer of 2012-2013, a conservative number of 15,970 flying foxes are estimated to have died due to heat-stress.

Table 1: Reported numbers of Grey-headed flying foxes that have died during heat-stress events in Adelaide (L Collins 2020, pers. comm.)

Season	Event dates	Approximate Camp count	Maximum Temperature /^{O}C	Approximate Mortality Count	Approximate Incidence risk
Summer 2012-13	4-18 Jan 2013	unknown	44.1	90	-
Summer 2013-14	18-21 Dec 2013	unknown	43.4	22	-
	14-24 Jan 2014	unknown	44.2	158	-
	14 Feb 2014	unknown	44.7	9	-
Summer 2014-15	2-6 Jan 2015	3,000	44.1	555	18.50%
	1 Feb 2015	2,500	41.6	51	2.00%
Summer 2017-18	6-7 Jan 2018	13,000	42.3	12	0.09%
	18-19 Jan 2018	13,000	42.2	73	0.60%
	24 Jan 2019	20,500	46.6	4,000	19.50%
Summer 2018-19	20 Nov 2019	25,000	41.6	2,000	8.00%
Summer 2019-20	17-20 Dec 2019	23,000	45.3	9,000	36.10%

1.6.4.2 Electrocution and entanglement

Historically, animals are involved in 6-7% of power outages in the distribution network, with birds responsible for about 4% and bats and possums comprising about 1% each (Energy Source and Distribution, 2018). The number of outages caused by animals is increasing and that increase is specifically related to the arrival and population growth of the Grey-headed flying fox camp in Adelaide particularly over the period between 2016 and 2021. In search of food, flying foxes often get entangled in power lines, killing the individual. Occasionally, offspring attached to mothers can still be alive (Energy Source and Distribution, 2018). The bats, which have a wide wingspan (over one metre) (Fig.6), can cause a power outage when they make contact between power lines and pole top equipment, including insulators, transformers and switches or when trapped by lightning arrestors. Power authorities need to remove the dead animal and restore power which can be disruptive to the electricity supply to affected areas. The power authorities look for spatial patterns in the outages, so where there are repeat incidents involving animals, they target solutions aimed at reducing the likelihood of further outages at that location (Energy Source and Distribution, 2018). Reports of entanglement of flying foxes in barbed-wire and also poor-quality netting has increased in

South Australia (L Collins 2021, pers.comm.), which may relate to the increasing numbers reported in the state. Often animals are found dead or so badly injured that euthanasia is necessary.

Fig 6: Flying fox electrocuted on power lines (Source: John Martin)

1.6.4.3 Plane strikes

Plane strikes caused by collision with flying foxes are common (Parsons et al, 2009). There have been three known plane strikes (Fig 7) associated with flying foxes, twice at Parafield airport in the northern suburbs of Adelaide and once at Adelaide international airport between 2016 and 2018 (J Van Weenen 2019, pers. comm.). No substantial damage was done to the aircraft and its passengers, but mitigation strategies to minimise the risk of future strikes is being considered by the South Australian Department of Environment and Water and aviation authorities.

Fig 7: Damage to a light aeroplane, Socata TB-10 Tobago, caused

by a collision with a Grey-headed flying fox (July 17, 2017)

1.6.4.4 Primary producer concerns

The Adelaide hills, east of the metropolitan area, are home to important primary production of apples, pears and cherries. Primary producers have been concerned for their crops with the presence of Grey-headed flying foxes nearby but, so far, their presence has caused little impact (Billington and Bailey, 2015). This is in part due to producers having increasingly used exclusion netting to protect crops from bird damage (J Van Weenen 2020, pers. comm.). While costs of netting can be prohibitive, e.g. AUD $43-72,000 per hectare (Billington and Bailey, 2015), some of the cost is recovered with enhanced productivity because of bird and bat exclusion as well as reduced heat, hail, sunburn and windburn.

1.6.4.5 Public health concerns

Since the arrival of Grey-headed flying foxes in the Adelaide region, concerns have been raised that the bat camp may constitute a biohazard reservoir for the public and domestic animals. Since the report of ABLV in the camp in 2012 (Cox-Witton, 2019), twenty-six additional individuals have been opportunistically screened and all returned negative results (Cox-Witton, 2019). Uncertainty remains about the endemicity of ABLV in the Adelaide camp and the carriage of other zoonotic viruses. Furthermore, phylogenetic analysis of a new Hendra-like virus, of unknown pathogenicity detected in Grey-headed flying foxes in January 2013 at the University of Adelaide School of Animal and Veterinary Sciences, Roseworthy, Adelaide, indicated a henipavirus variant which is yet to be fully evaluated.

1.7 Thesis aims

The aims of this thesis were to investigate the movement, dietary and viral potential of the Grey-headed Flying foxes that have established a permanent camp in Adelaide, expanding the distribution range of the species. Specifically, I wished to understand why they have come to Adelaide; is it due to 'pull' factors (being attracted to Adelaide) or 'push' factors (loss of resources in their former range). While it is not within the remit of this PhD to assess 'push factors', I wanted to explore potential 'pull factors' and learn how they use their new landscape, what foraging species they utilise during the year and what emerging infectious diseases of zoonotic importance have accompanied the flying foxes into the Adelaide region.

Specifically, I aimed:

1. **to characterise the foraging resources and habits of the Adelaide's Grey-headed flying foxes that supported the establishment of a permanent camp;**

2. **to investigate the potential public health threats associated with the establishment of a permanent Grey-headed flying fox population in an urban environment.**

Chapter 2 addresses aim number 1 and 2 by attaching GPS collars to selected flying foxes and monitoring their movements in the Adelaide district during Spring 2015. We investigated foraging range area and utilisation distribution, the most frequently visited foraging sites, the visitation frequency and duration of the most frequently visited sites and ground truthed forage plants from these sites.

Chapter 3 addresses aim number 1 using DNA metabarcoding of faeces collected from flying foxes captured in the camp. We used two plant chloroplast DNA primers; *rbcL* and *ndhJ* to investigate the taxonomy of foraging plants eaten by the flying foxes and compare with covariates such as season.

Chapters 4 and 5 address aim number 2 by testing collected serum for exposure to several bat vectored viruses including Hendra virus, Tioman virus, Cedar virus, Australian bat lyssavirus, Ebola Zaire virus, Middle Eastern respiratory syndrome virus and Severe acute respiratory syndrome virus, from flying foxes sampled over six survey events across three years. We investigated the serostatus of each of the viruses with covariates such as season, year, age, sex, weight and body condition.

Chapter 6 summarises my findings and highlights areas of research that should be continued to further understand the ecology and zoonotic potential of the Grey-headed flying foxes that have become established in Adelaide.

1.8 References

Allen T, Murray KA, Zambrana-Torrelio C, Morse SS, Rondinini C, Di Marco M, Breit N, Olival KJ, Daszak P. (2017). Global hotspots and correlates of emerging zoonotic diseases. *Nature Communications,* 8 :1124. doi: 10.1038/s41467-017-00923-8

Ancillotto L, Santini L, Ranc N, Maiorano L, Russo D. (2016). Extraordinary range expansion in a common bat: The potential roles of climate change and urbanisation. *The Science of Nature*, 103:15. doi: 10.1007/s00114-016-1334-7.

Andersen KG, Rambaut A, Lipkin WI, Holmes EC and Garry RF. (2020). The proximal origin of SARS-CoV-2. *Nature Medicine*, 26, 450–452. doi: 10.1038/s41591-020-0820-9

Annand E and Reid P. (2014). Clinical review of two fatal equine cases of infection with the insectivorous bat strain of Australian bat lyssavirus. *Australian Veterinary Journal*, 92: 324–332. doi: 10.1111/avj.12227

Anthony SJ, Johnson CK, Greig DJ, Kramer S, Che X, Wells H, Hicks AL, Joly DO, Wolfe ND, Daszak P, Karesh W, Lipkin WI, Morse SS, PREDICT Consortium, Mazet JAK, Goldstein T. (2017). Global patterns in coronavirus diversity. *Virus Evolution*, 3:1. doi: 10.1093/ve/vex012.

Baguette M and Van Dyck H. (2007). Landscape connectivity and animal behavior: Functional grain as a key determinant for dispersal. *Landscape Ecology*, 22, 1117–1129. doi: 10.1007/s10980-007-9108-4

Barr JA, Smith C, Marsh GA, Field H, Wang L-F. (2012). Evidence of bat origin for Menangle virus, a zoonotic paramyxovirus first isolated from diseased pigs. *Journal of General Virology,* 93, 2590–2594. doi: 10.1099/vir.0.045385-0

Banerjee A, Kulcsar K, Misra V, Frieman M, Mossman K. (2019). Bats and Coronaviruses. *Viruses*, 11:41. doi: 10.3390/v11010041

Benton TG and Bowler DE (2012). Linking dispersal to spatial dynamics. In J. Clobert, M. Baguette, T. G. Benton, & J. M. Bullock (Eds.), Dispersal ecology and evolution (pp. 251–265). Oxford, UK: Oxford University Press. doi: 10.1093/acprof:oso/9780199608898.001.0001

Bempong NF, De Castañeda RR, Schütte S, Bolon I, Keiser O, Escher G, Flahault A. (2019). Precision Global Health - The case of Ebola: a scoping review. *Journal of Global Health,* 9:1. doi: 10.7189/jogh.09.010404

Billington K and Bailey H. (2015). Evaluating the netting of apple orchards in the Adelaide Hills with the development of a business case tool for growers. Report for Apple and Pear Growers Association of South Australia. Available here. Accessed December 2020.

Birt P. (2005). Mutualistic interactions between the nectar feeding little red flying fox *Pteropus scapulatus* (Chiroptera: Pteropodidae) and flowering eucalypts (Myrtaceae): habitat utilisation and pollination. PhD dissertation. University of Queensland, Queensland.

Boer MM, de Dios VR, Bradstock RA. (2020). Unprecedented burn area of Australian mega forest fires. *Nature Climate Change*, 10(3):171–2. doi: 10.1038/s41558-020-0716-1

Bradshaw CJ. Little left to lose: deforestation and forest degradation in Australia since European colonization. *Journal of Plant Ecology*, 2012; 5(1):109–20. doi: 10.1093/jpe/rtr038

Bradshaw WE & Holzapfel CM. (2006). Evolutionary response to rapid climate change. *Science*,312, 1477–1478. doi: 10.1126/science.1127000

Breed AC, Breed MF, Meers J, Field HE. (2011). Evidence of endemic Hendra virus infection in flying foxes (*Pteropus conspicillatus*) - implications for disease risk management. *PLoS ONE* 6: e28816. doi: 28810.21371/journal.pone.0028816

Business Queensland. (2021). Summary of Hendra virus incidents in horses. Available at https://www.business.qld.gov.au/industries/service-industries-professionals/service-industries/veterinarysurgeons/guidelines-hendra/incident-summary (Accessed 5 February 2021).

Burgess SC, Baskett ML, Grosberg RK, Morgan SG & Strathmann, RR. (2016). When is dispersal for dispersal? Unifying marine and terrestrial perspectives. *Biological Reviews*, 91, 867–882. doi: 10.1111/brv.12198

Burton OJ and Travis JMJ. (2008). The frequency of fitness peak shifts is increased at expanding range margins due to mutation surfing. *Genetics* 179, 941–950. doi: 10.1534/genetics.108.087890

Byrne AW, O'Keeffe J, Buesscheng CD, Newman C. (2019). Push and pull factors driving movement in a social mammal: context dependent behavioral plasticity at the landscape scale. Current Zoology, 65 (5): 517-525. doi: 10.1093/cz/zoy081

Calisher CH, Childs JE, Field HE, Holmes KV and Schountz T. (2006). Bats: important reservoir hosts of emerging viruses. *Clinical Microbiology Reviews,* 19, 531–545. doi: 10.1128/CMR.00017-06

Chatterjee P. (2018). Nipah virus outbreak in India. *Lancet*, Vol 391, 2200. doi: 10.1016/S0140-6736(18)31252-2

Chant K, Chan R, Smith M, Dwyer DE, Kirkland P. and The NSW Expert Group. (1998). Probable human infection with a newly described virus in the family Paramyxoviridae. *Emerging Infectious Diseases,* 4, 273–275. doi: 10.3201/eid0402.980215

Chowell G and Nishiura H. (2014). Transmission dynamics and control of Ebola virus disease (EVD): a review. *BMC Medicine,* 12**,** 196. doi: 10.1186/s12916-014-0196-0

Chua KB**,** Goh KJ, Wong KT, Kamarulzaman A, Tan PS, Ksiazek TG, Zaki SR, Paul G, Lam K and Tan CT. **(**1999). Fatal encephalitis due to Nipah virus among pig-farmers in Malaysia. *Lancet,* 354**:** 1257-*1259.* doi: 10.1016/S0140-6736(99)04299-3

Chua KB, Bellini W, Rota P, Harcourt B, Tamin A, Lam S, Ksiazek T, Rollin P, Zaki S, Shieh W-J, Goldsmith C, Gubler D, Roehrig J, Eaton B, Gould A, Olson J, Field H, Daniels P, Ling A, Peters C, Anderson L and Mahy B. (2000). Nipah virus: a recently emergent deadly paramyxovirus. *Science,* 288:1432-1435. doi: 10.1126/science.288.5470.1432

Chua KB**,** Koh C, Hooi P, Wee K, Khong J, Chua B, Chan Y, Lim M, and Lam S. *(*2002*)*. Isolation of Nipah virus from Malaysian Island flying foxes. *Microbes and Infection,* 4**:** 145-*151.* doi: 10.1016/S1286-4579(01)01522-2

Cote J, Bestion E, Jacob S, Travis J, Legrand D & Baguette M. (2017). Evolution of dispersal strategies and dispersal syndromes in fragmented landscapes. *Ecography*, 40, 56–73. doi: 10. 1111/ecog.02538

Cote J, Bocedi G, Debeffe L, Chudzińska ME, Weigang HC, Dytham, Hewison AJ. (2017). Behavioural synchronization of large-scale animal movements–disperse alone, but migrate together? *Biological Reviews*, 92, 1275–1296. doi: 10.1111/brv. 12279

Cowan T, Purich A, Perkins S, Pezza A, Boschat G. & Sadler K. (2014). More frequent, longer, and hotter heatwaves for Australia in the twenty-first century. *Journal of Climate,* 27,5851–5871. doi: 10.1175/JCLI-D-14-00092.1

Cox-Witton K. Australian bat lyssavirus Bat Stats. Wildlife Health Australia website. Available here. Sourced December 2020.

Crone EE, Brown LM, Hodgson JA, Lutscher F and Schultz CB. (2019). Faster movement in nonhabitat matrix promotes range shifts in heterogeneous landscapes. *Ecology*, 100(7): e02701. doi: 10.1002/ecy.2701

Cui J, Han N, Streicker D, Li G, Tang X, Shi Z, Hu Z, Zhao G, Fontanet A, Guan Y, Wang L-F, Jones G, Field HE, Daszak P, Zhang S. (2007). Evolutionary relationships between bat coronaviruses and their hosts. *Emerging Infectious Diseases,* 13:1526–1532. doi: 10.3201/eid1310.070448

de Groot RJ, Baker SC, Baric RS, Brown CS, Drosten C, Enjuanes L, Fouchier RA, Galiano M, Gorbalenya AE, Memish ZA, Perlman S, Poon LL, Snijder EJ, Stephens GM, Woo PC, Zaki AM, Zambon M, Ziebuhr J. (2013). Middle east respiratory syndrome coronavirus (MERS-CoV): announcement of the coronavirus study group. *Journal of Virology*, 87:7790–7792. doi: 10.1128/JVI.01244-13

Dong E, Du H, Gardner L. (2020) An interactive web-based dashboard to track COVID-19 in real time. *Lancet Infectious Diseases*, 20(5):533-534. doi: 10.1016/S1473-3099(20)30120-1

Drexler JF, Corman VM, Gloza-Rausch F, Seebens A, Annan A, Ipsen A, Kruppa T, Muller MA, Kalko EKV, Adu-Sarkodie Y, Oppong S, Drosten C. (2009). Henipavirus RNA in African Bats. *PLoS ONE* 4 (7). doi: 10.1371/journal.pone.0006367

Drexler JF, Corman VM, Müller MA, Maganga GD, Vallo P, Binger T, Gloza-Rausch F, Rasche A, Yordanov S, Seebens A, Oppong S, Adu-Sarkodie Y, Pongombo C, Lukashev AN, Schmidt-Chanasit J, Stocker A, Carneiro AJ, Erbar S, Maisner A, Fronhoff F, Buettner R, Kalko EK, Kruppa T, Franke CR, Kallies R, Yandoko ER, Herrler G, Reusken C, Hassanin A, Kruger DH, Matthee S, Ulrich RG, Leroy EM, Drosten C (2012). Bats host major mammalian paramyxoviruses. *Nature Communications*, 3:796. doi: 10.1038/ncomms1796

Duncan AM, Baker GB, Montgomery N. and Natural Heritage Trust. (1999) The action plan for Australian bats. Canberra: Natural Heritage Trust. https://catalogue.nla.gov.au/Record/3427263?lookfor=author:%22Duncan,%20Anne%&offset=7&max=19. Accessed November 2020.

Eby P. (1991). Seasonal movements of Grey-headed flying foxes, *Pteropus poliocephalus* (Chiroptera: Pteropodidae), from two maternity camps in northern New South Wales. *Wildlife Research,* 18: 547–59. doi: 10.1071/WR9910547

Eby P. (1996). Interactions between the Grey-headed flying fox *Pteropus poliocephalus* (Chiroptera: Pteropodidae) and its diet plants – seasonal movements and seed dispersal. PhD Thesis, University of New England, Armidale.

Eby P. (1998). An analysis of diet specialization in frugivorous *Pteropus poliocephalus* (Megachiroptera) in Australian subtropical rainforest. *Australian Journal of Ecology*. 23: 443–456. doi: 10.1111/j.1442-9993.1998.tb00752.x

Eby P, Richards G, Collins L and Parry-Jones K. (1999). The distribution, abundance and vulnerability to population reduction of a nomadic nectarivore, the Grey-headed flying fox, *Pteropus poliocephalus*, in New South Wales, during a period of resource concentration. *Australian Zoologist*, 31, 240–53. doi: 10.7882/AZ.1999.024

Eby P. (2002). National Count of Grey-headed Flying foxes July 27 & 28. Report to Environment Australia, Queensland Parks and Wildlife Service, NSW National Parks and Wildlife Service, Victoria Dept Natural Resources and Environment.

Eby P and Lunney D. (2002). Managing the Grey-headed Flying fox *Pteropus poliocephalus* as a threatened species in NSW: adjusting to a long-term vision. In: Eby P, Lunney D, Ed. *Managing the Grey-headed flying fox as a threatened species in NSW*. Mosman, NSW: Royal Zoological Society of NSW. 273–284.

Eby P. (2004) 'National count of Grey-headed flying foxes April 3 & 4, 2004.' A report to the Department of Environment and Heritage, Canberra

Edson D, Field H, McMichael L, Vidgen M, Goldspink L, Broos A, Melville D, Kristoffersen J, de Jong C, McLaughlin A, Davis R, Kung N, Jordan D, Kirkland D, Smith C. (2015). Routes of Hendra virus excretion in naturally infected flying foxes: implications for viral transmission and spillover risk. *PLoS ONE*. 10 (10): e0140670. doi: 10.1371/journal.pone.0140670

Edson D, Peel AJ, Huth L, Mayer DG, Vidgen ME, McMichael L, Broos A, et al, Melville D, Kristoffersen J, de Jong C, McLaughlin A, Field HE. (2019). Time of year, age class and body condition predict Hendra virus infection in Australian black flying foxes (*Pteropus alecto*). *Epidemiology and Infection*, Volume 147, e240. doi: 10.1017/S0950268819001237.

Energy Source and Distribution. Flying foxes causing power outages in Adelaide 2018. Available from: https://www.esdnews.com.au/flying foxes-causing-power-outages-adelaide/. (Accessed December 1 2020)

Everman E and Klawinski P. (2013) Human-facilitate jump dispersal of a non-native frog species in Hawa'ii Island. *Journal of Biogeography*, 40: 1961-1970. doi: 10.1111/jbi.12146.

Feldmann H, Czub M, Jones, S, Dick D, Garbutt M, Grolla A, Artsob H. (2002). Emerging and re-emerging infectious diseases. *Medical Microbiology and Immunology,* 191, 63–74. doi: 10.1007/s00430-002-0122-5

Field HE, Young P, Yob JM, Mills J, Hall L, and Mackenzie JS. (2001). The natural history of Hendra and Nipah viruses. *Microbes and Infection* 3:307-314. doi: 10.1016/S1286-4579(01)01384-3

Field HE. (2004). The ecology of Hendra virus and Australian bat lyssavirus. PhD Thesis, The University of Queensland, Brisbane.

Field HE, de Jong C, Melville D, Smith C, Smith I, Broos A, Kung YH, McLaughlin A, Zeddeman A. (2011). Hendra virus infection dynamics in Australian fruit bats. *PLoS ONE,* 6: e28678. doi: 10.1371/journal.pone.0028678

Field HE, Smith CS, de Jong CE, Melville D, Broos A, Kung N, Thompson J, Dechmann DK. (2016). Landscape utilisation, animal behaviour and Hendra virus risk. *EcoHealth*, 13(1):26–38. doi: 10.1007/s10393-015-1066-8

Francis JR, Nourse C, Vaska VL, Calvert S, Northill JA, McCall B and Mattke AC. (2014). Australian Bat Lyssavirus in a Child: The First Reported Case. *Pediatrics*, 133; e1063. doi: 10.1542/peds.2013-1782

Fujita MS, Tuttle MD. (1991). Flying foxes (Chiroptera: Pteropodidae): threatened animals of key ecological and economic importance. *Conservation Biology*, 5, 455–463.

Garnett ST, Whybird OA, Spencer HG. (1999). Conservation status of the Spectacled Flying fox *Pteropus conspicillatus*. *Australian Zoologist*, 31:38-54. doi: 10.7882/AZ.1999.006

Ge XY, Li JL, Yang XL, Chmura AA, Zhu G, Epstein JH, Mazet JK, Hu B, Zhang W, Peng C, Zhang Y-J, Luo C, Tan B, Wang N, Zhu Y, Crameri G, Zhang S-Y, Wang L-F, Daszak P, Shi Z-L. (2013). Isolation and characterization of a bat SARS-like coronavirus that uses the ACE2 receptor. *Nature,* 503:535–8. doi: 10.1038/nature12711

Goh KJ, Tan TC, Chew NK, Tan PS, Karmarulzaman A, Sarji SA, Wong KT, Abdullah BJJ, Chua KB, and Lam SK. (2000). Clinical features of Nipah virus encephalitis among pig farmers in Malaysia. *New England Journal of Medicine*, 342**:**1229-*1235.* doi: 10.1056/NEJM200004273421701

Gorbunova V, Seluanov, A, Kennedy BK. (2020). The World Goes Bats: Living longer and tolerating viruses. *Cell Metabolism, 32*, 31–43. doi: 10.1016/j.cmet.2020.06.013

Guan Y, Zheng BJ, He YQ, Liu XL, Zhuang ZX, Cheung CL, Luo SW, Li PH, Zhang LJ, Guan YJ, Butt KM, Wong KL, Chan KW, Lim W, Shortridge KF, Yuen KY, Peiris JS, and Poon LL. (2003). Isolation and characterization of viruses related to SARS coronavirus from animals in southern China. *Science,* 302:276-*278.* doi: 10.1126/science.1087139

Gurley ES, Montgomery JM, Hossain MJ, Bell M, Kalam AK, Islam MR, Molla MAR, Carroll DS, Ksiazek TG, Rota PA, Lowe L, Comer JA, Rollin P, Czub M, Grollla A, Feldmann H, Luby SP, Woodward JL, Breiman RF. (2007). Person-to-person transmission of Nipah virus in a Bangladeshi community. *Emerging Infectious Diseases*, 13(7): 1031-1037. doi: 10.3201/eid1307.061128

Guyatt KJ, Twin J, Davis P, Holmes EC, Smith GA, Smith IL, Mackenzie JS and Young PL. (2003). A molecular epidemiological study of Australian bat lyssavirus. *Journal of General Virology,* 84:485–496. doi: 10.1099/vir.0.18652-0

Hall LS and Richards G. (2000). Flying foxes: Fruit and Blossom Bats of Australia. University of New South Wales Press, Sydney

Hall LS. (2002). Management of flying fox camps: What have we learnt in the last twenty-five years? Managing the Grey-headed flying fox as a threatened species in NSW. P. Eby and Lunney, D. Sydney, Royal Zoological Society of NSW: 215-224.

Halpin K, Young P and Field HE. (1996). Identification of likely natural hosts for equine morbillivirus. Communicable Diseases Intelligence, 20 (22):476

Halpin K, Young PL, Field HE, MacKenzie JS. (1999). Newly discovered viruses of flying foxes. *Veterinary Microbiology,* 68(1-2): 83-87. doi: 10.1016/S0378-1135(99)00063-2

Halpin K, Young PL, Field HE, Mackenzie JS. (2000). Isolation of Hendra virus from pteropid bats: a natural reservoir of Hendra virus. *Journal of General Virology*, 81:1927–32. doi: 10.1099/0022-1317-81-8-1927

Hu B, Zeng LP, Yang XL, Ge XY, Zhang W, Li B, Xie J-Z, Shen Y-Z, Wang N, Luo D-S, Zheng X-S, Wang M-N, Daszak P, Wang L-F, Cui J, Shi Z-L. (2017). Discovery of a rich gene pool of bat SARS-related coronaviruses provides new insights into the origin of SARS coronavirus. *PLoS Pathogens*, 13. doi: 10.1371/journal.ppat.1006698

ICTV Taxonomy History: Cornidovirineae. Available online: https://talk.ictvonline.org/taxonomy/p/taxonomy-history?taxnode_id=20186105. (Accessed on 1 December 2020).

Johara M, Field H, Rashdi A, Morrissy C, vanderHeide B, Rota P, Azri A, White J, Daniels P, Jamaluddin A, and Ksiazek T. (2001). Serological evidence of infection with Nipah virus in bats (order Chiroptera) in Peninsular Malaysia. *Emerging Infectious Diseases*, 7:439-441. doi: 10.3201/eid0703.010312

Jones KE and MacLarnon A. (2001). Bat life histories: testing models of mammalian life-history evolution. *Evolutionary Ecology Research,* 3, 465–476.

Jordano P. (2017). What is long-distance dispersal? And a taxonomy of dispersal events. *Journal of Ecology*, 105: 75-84. doi: 10.1111/1365-2745.12690

Karsten M, van Vuuren BJ, Barnaud A, Terblanche JS. (2013). Population genetics of Ceratitis capitata in South Africa: Implications for Dispersal and Pest management. *PLoS ONE*, 8 (1): e54281. doi: 10.1371/journal.pone.0054281

Krebs CJ. (2009). In Ecology, the Experimental Analysis of Distribution and Abundance. 6th ed. Pearson Benjamin Cummings: San Franscisco.

Laing ED, Mendenhall IH, Linster M, Low DHW, Chen Y, Yan, L, Sterling SL, Borthwick S, Neves, ES, Lim, JSL, Skiles M, Lee BPYH, Wang L_F, Broder CC, Smith GJD. (2018). Serologic evidence of fruit bat exposure to filoviruses, Singapore, 2011-2016. *Emerging Infectious Diseases,* 24:1 122-126. doi: 10.3201/eid2401.170401

Latinne A, Hu B, Olival KJ, Zhu G, Zhang L, Li H, Chmura AA, Field HE, Zambran-Torrelio C, Epstein JH, Li B, Zhang W, Wang L-F, Shi Z-L, Daszak P. (2020). Origin and cross-species transmission of bat coronaviruses in China. *Nature Communications*, 11: 4235. doi: 10.1038/s41467-020-17687-3

Laupland KB, Valiquette L. (2014). Ebola Virus Disease. *Canadian Journal of Infectious Disease and Medical Microbiology*, 25(3): 128–129. doi: 10.1155/2014/527378

Lau SKP, Zhang L, Luk HKH, Xiong L, Peng X, Li KSM, He X, Zhao PS, Fan RYY, Wong ACP, Ahmed SS, Cai J-P, Chan JFW, Sun Y, Jin D, Lau TCK, Kok RKH, Li W, Yuen K-Y, Woo PCY. (2018). Receptor usage of a novel bat lineage C betacoronavirus reveals evolution of MERS-related coronavirus spike proteins for human DPP4 binding. *Journal of Infectious Disease,* 218: 2. doi: 10.1093/infdis/jiy018

Lenoir J, Svenning JC. (2015). Climate-related range shifts—a global multidimensional synthesis and new research directions. *Ecography*, *38:* 15– 28. doi: 10.1111/ecog.00967

Leroy EM, Kumulungui B, Pourrut X, Rouquet P, Hassanin A, Yaba P, Delicat A, Paweska JT, Gonzalez JP and Swanepoel R. (2005). Fruit bats as reservoirs of Ebola virus. *Nature,* 438; 575–576. doi: 10.1038/438575a

Luis AD, Hayman DTS, O'Shea TJ, Cryan PM, Gilbert AT, Pulliam JRC, Mills JM, Timonin ME, Willis CKR, Cunningham AA, Fooks AR, Rupprecht CE, Wood JLN, Webb CT. (2013). A comparison of bats and rodents as reservoirs of zoonotic viruses: are bats special? *Philosophical Transactions Royal Society of London B: Biological Sciences,* 280:20122753. doi: 10.1098/rspb.2012.2753

Lunney D and Moon C. (1997). Flying foxes and their camps in the remnant rainforests of north-east New South Wales. Australia's Everchanging Forests III Centre for Resource and Environmental Studies. Canberra: Australian National University; p. 247–77.

Markus N and Hall L. (2004). Foraging behaviour of the black flying fox (*Pteropus alecto*) in the urban landscape of Brisbane, Queensland. *Wildlife Research*, 31(3):345–55. doi: 10.1071/WR01117

Marsh GA, de Jong C, Barr JA, Tachedjian M, Smith C, Middleton D, Yu M, Todd S, Foord AJ, Haring V, Payne J, Robinson R, Broz I, Crameri G, Field HE, Wang L-F. (2012). Cedar Virus: A Novel Henipavirus Isolated from Australian Bats. *PLoS Pathogens,* 8 (8): e1002836. doi: 10.1371/journal.ppat.1002836

Martin GA, Yanez-Arenas C, Roberts BJ, Chen C, Plowright RK, Webb RJ, Skerratt LF. (2016). Climatic suitability influences species specific abundance patterns of Australian flying foxes and risk of Hendra virus spillover. *One Health*, 2, 115-121. doi: 10.1016/j.onehlt.2016.07.004

McDonald-Madden E, Schreiber ESG, Forsyth DM, Choquenot D and Clancy TF. (2005). Factors affecting Grey-headed flying fox (*Pteropus poliocephalus*: Pteropodidae) foraging in the Melbourne metropolitan area, Australia. *Austral Ecology*, 30: 600-608. doi: 10.1111/j.1442-9993.2005.01492.x

McFarlane RA, Sleigh A and McMichael AJ. (2012). Synanthropy of wild mammals as a determinant of emerging infectious diseases in the Asian and Australasian region. *EcoHealth*, 9, 24–35. doi: 10.1007/s10393-012-0763-9

McFarlane RA. (2015). In Health of people, places and planet. Ed CD Butler, J Dixon, AG Capon. Australia National University Press.

McIlwee AP and Martin L. (2002). On the intrinsic capacity for increase of Australian flying foxes (*Pteropus spp., Megachiroptera*). *Australian Zoologist*, 32: 1, pp. 76-100. doi: 10.7882/AZ.2002.008

Memish ZA, Mishra N, Olival KJ, Fagbo SF, Kapoor V, Epstein JH, Alhakeem R, Durosinloun A, Al Asmari M, Islam A, Kapoor A, Briese T, Daszak P, Al Rabeeah AA, Lipkin WI. (2013). Middle East respiratory syndrome coronavirus in bats, Saudi Arabia. *Emerging Infectious Disease*, 19, 1819–1823. doi: 10.3201/eid1911.131172

Murray L, Rogers R, Selvey L, Selleck P, Hyatt A, Gould A, Gleeson L, Hooper P and Westbury H (1995). A novel Morbillivirus pneumonia of horses and its transmission to humans. *Emerging Infectious Diseases*, 1 (1): 31-33. Doi: 10.3201/eid0101.950107.

Negredo A, Palacios G, Vazquez-Moron S, Gonzalez F, Dopazo H, Molero F. (2011). Discovery of an Ebolavirus-like filovirus in Europe. *PLoS Pathogens,* 7:e1002304. doi: 10.1371/journal.ppat.1002304

Nelson JE. (1965). Movements of Australian flying foxes (Pteropodidae: Megachiroptera). *Australian Journal of Zoology* 13, 53–74. doi: 10.1071/ZO9650053

Olival KJ, Islam A, Yu M, Anthony SJ, Epstein JH, Khan SA, Khan SU, Crameri G, Wang L-F, Lipkin WI, Luby SP, Daszak P. (2013). Ebola virus antibodies in fruit bats, Bangladesh. *Emerging Infectious Disease*, 19, 270–273. doi: 10.3201/eid1902.120524

Olival KJ and Hayman DTS. (2014). Filoviruses in Bats: Current Knowledge and Future Directions. *Viruses*, 6, 1759-1788. doi:10.3390/v6041759

Parris K and Hazell D. (2005). Biotic effects of climate change in urban environments: The case of the Grey-headed flying fox (Pteropus poliocephalus) in Melbourne, Australia. *Biological Conservation* 124: 267–276. doi: 10.1016/j.biocon.2005.01.035

Parry-Jones KA and Augee ML. (1992). Movements of Grey-headed flying foxes (*Pteropus poliocephalus*) to and from a colony site on the central coast of New South Wales. *Wildlife Research,* 19: 331–340. doi: 10.1071/wr9920331

Parry-Jones, K.A. and M.L. Augee. (2001). Factors affecting the occupation of a colony site in Sydney, New South Wales by the Grey-headed flying fox (Pteropodidae). *Austral Ecology*, 26: 47–55. doi: 10.1111/j.1442-9993.2001.01072.pp.x

Parsons JG, Blair D, Luly J, Robson SKA. (2009). Bat Strikes in the Australian Aviation Industry. *Journal of Wildlife Management* ;73(4):526-9. doi: 10.2193/2008-268

Parsons JG, Van Der Wal J, Robson SKA, Shilton LA. (2010). The implications of sympatry in the spectacled and Grey headed flying fox, *Pteropus conspicillatus* and *P. poliocephalus* (Chiroptera: Pteropodidae). *Acta Chiropterologica*, 12: 301–309. doi.org/10.3161/150811010X537882

Ratnayake HU, MR Kearney, P Govekar, D Karoly and Welbergen JA. (2019). Forecasting wildlife die-offs from extreme heat events. *Animal Conservation,* 22: 386–395. doi: 10.1111/acv.12476

Peel AJ, Wells K, Giles J, Boyd V, Burroughs A, Edson D, Crameri G, Baker ML, Field HE, Wang L-F, McCullum H, Plowright RK, Clark N. (2019). Synchronous shedding of multiple paramyxoviruses coincides with peak periods of Hendra virus spillover. *Emerging Microbes and Infections,* 8: 1314-1323. doi: 10.1080/22221751.2019.1661217

Philbey AW, Kirkland PD, Ross AD, Davis RJ, Gleeson AB, Love RJ, Daniels PW, Gould AR, and Hyatt AD. (1998). An apparently new virus (family Paramyxoviridae) infectious for pigs, humans, and fruit bats. *Emerging Infectious Disease,* 4:269-271. doi: 10.3201/eid0402.980214

Plowright RK, P Eby, PJ Hudson, IL Smith, DA Westcott, Bryden WL, Middleton D, Reid PA, McFarlane RA, Martin G, Tabor GM, Skerratt LF, Anderson DL, Crameri G, Quammen D, Jordan D, Freeman P, Wang L-F, Epstein JH, Marsh GA, Kung NY and McCallum H. (2015). Ecological dynamics of emerging bat virus spillover. *Proceedings of the Royal Society B: Biological Sciences*, 282: 20142124. doi: 10.1098/rspb.2014.2124

Pourrut X, Souris M, Towner JS, Rollin PE, Nichol ST, Gonzalez JP, Leroy E. (2009). Large serological survey showing cocirculation of Ebola and Marburg viruses in Gabonese bat populations, and a high seroprevalence of both viruses in *Rousettus aegyptiacus*. *BMC Infectious Diseases*, 9. doi:10.1186/1471-2334-9-159.

Prada D, Boyd V, Baker M, Jackson B, O'Dea M. (2019). Insights into Australian Bat Lyssavirus in Insectivorous Bats of Western Australia. *Tropical Medicine and Infectious Disease*, 4: 46. doi: 10.3390/tropicalmed4010046

Ratcliffe FN. 1931. The flying fox (Pteropus) in Australia. *CSIR Bulletin*, Melbourne. 53pp.

Richardson S, Mill AC, Davis D, Jam D and Ward AI. (2020). A systematic review of adaptive wildlife management for the control of invasive, non-native mammals, and other human–wildlife conflicts. *Mammal Review*, **50,** 147– 156. doi: 10.1111/mam.12182

Roberts BJ, Catterall CP, Eby P and Kanowski J. (2012). Long-distance and frequent movements of the flying fox *Pteropus poliocephalus*: implications for management. *PLoS ONE,* 7: e42532. doi: 10.1371/journal.pone.0042532

Root TL, Price JT, Hall KR, Schneider SH, Rosenzweig C and Pounds JA. (2003). Fingerprints of global warming on wild animals and plants. *Nature*, 421, 57–60. doi: doi.org/10.1038/nature01333

Sanchez A, Khan AS, ZakiS R, Nabel GJ, Ksiazek T, Peters C. (2001). Filoviridae: Marburg and Ebola viruses. In: Knipe DM, Howley PM, editors. *Fields virology*, 4th ed. Philadelphia: Lippincott Williams & Wilkins; p. 1279–304

Schmelitschek E, French K and Parry-Jones K. (2009). Fruit availability and utilisation by Grey -headed flying foxes (Pteropodidae: *Pteropus poliocephalus*) in a human-modified environment on the south coast of New South Wales, Australia. *Wildlife Research*, 36: 592-600. doi: 10.1071/WR08169

Simmons NB. (2005). Genus *Pteropus*. In DE Wilson and DM Reeder (Eds.). Mammal species of the world: A taxonomic and geographic reference (3rd ed, pp. 334-346, Johns Hopkins University Press, Baltimore, Maryland, USA.

Shilton LA, Altringham JD, Compton SG, Whittaker RJ. (1999). Old World fruit bats can be long–distance seed dispersers through extended retention of viable seeds in the gut. *Proceedings of the Royal Society B: Biological Sciences*, 266 (1416) :219–23. doi: 10.1098/rspb.1999.0625

Soga M and Gaston KJ. (2020) The ecology of human-nature interactions. *Proceedings of the Royal Society B: Biological Sciences*. doi: 10.1098/rspb.2019.1882

Sohayati AR, Hassan L, Sharifah SH, Lazarus K, Zaini CM, Epstein JH, Shamsyul Naim N, Field HE, Arshad SS, Abdul Aziz J, Daszak P. (2011). Evidence for Nipah virus recrudescence and serological patterns of captive *Pteropus vampyrus. Epidemiology and Infection*, 139:1570–9. doi: 10.1017/S0950268811000550

Steffen W, Hughes L and Perkins S (2014). Heatwaves: Hotter, longer, more often. Australia: Climate Council of the Australian Ltd. Available here. Accessed 1 December, 2020.

Suarez AV and Tsutsui ND. (2008). The evolutionary consequences of biological invasions. *Molecular Ecology,* 17, 351–360. doi: 10.1111/j.1365-294X.2007.03456.x

Tait J, Perotto-Baldivieso HL, McKeown A, Westcott DA. (2014). Are flying foxes coming to town? Urbanisation of the spectacled flying fox (*Pteropus conspicillatus*) in Australia. *PLoS ONE*, 9(10). doi: 10.1371/journal.pone.0109810

Teagle S. (2002). Queensland Flying fox Consultative Committee – formation, outcomes and future strategies. In: Eby P, Lunney D, Ed. *Managing the Grey-headed flying fox as a threatened species in NSW*. Mosman, NSW: Royal Zoological Society of NSW. 273–284.

Teeling EC, Springer MS, Madsen O, Bates P, O'Brien SJ, Murphy WJ. (2005). A molecular phylogeny for bats illuminates biogeography and the fossil record. *Science,* 307 (5709):580-4. doi: 10.1126/science.1105113

Teitelbaum CS, Mueller T (2019). Beyond migration: Causes and Consequences of Nomadic Animal Movements. *Trends in Ecology and Evolution*, 34 (6): 569-581. doi: 10/1016/j.tree.2019.02.005

Tidemann CR, Kelson S and Jamieson G. (1997). Flying fox damage to orchard fruit in Australia: Incidence, extent and economic impact. *Australian Biologist,* 10: 179-186

Tidemann CR. (1999). Biology and management of the Grey-headed flying fox, Pteropus poliocephalus. *Acta Chiropterologica*, 1: 151–164. doi: 10.7882/fs.2002.04

Tidemann CR and Nelson J. (2004). Long-distance movements of the Grey-headed flying fox (Pteropus poliocephalus). *Journal of Zoology*, 263, 141–6. doi: 10.1017/S0952836904004960

Threatened Species Scientific Committee (2001a). Commonwealth Listing Advice on *Pteropus melanotus natalis* (Christmas Island Flying fox). Available here. In effect under the EPBC Act. Accessed 1 December 2020.

Threatened Species Scientific Committee (2001b). *Commonwealth Listing Advice on Pteropus conspicillatus* (Spectacled Flying fox). Available here. In effect under the EPBC Act. Accessed 1 December 2020.

Threatened Species Scientific Committee (2001c). *Commonwealth Listing Advice on Pteropus poliocephalus (*Grey-headed Flying fox*)*. Available here. In effect under the EPBC Act. Accessed 1 December 2020.

Towner JS, Amman BR, Sealy TK, Carroll SA, Comer JA, Kemp A, Swanerpoel R, Paddock CD, Balinandi S, Kristova ML, Formenty PBH, Albarion CG, Miller DM, Reed ZD, Kayiwa JT, Mills JN, Cannon DL, Greer PW, Byaruhanga E, Farnon EC, Katongole-Mbidde E, Downing R, Tappero JW, Zaki SR, Ksiazek TG, Nichol ST, Rollin PE. (2009). Isolation of genetically diverse Marburg viruses from Egyptian fruit bats. *PLoS Pathogens*, 5. doi: 10.1371/journal.ppat.1000536.

Tsagkogeorga G, Parker J, Stupka E, Cotton JA, Rossiter SJ. (2013). Phylogenomic analyses elucidate the evolutionary relationships of bats. *Current Biology,* 23, 2262–2267. doi: 10.1016/j.cub.2013.09.014

Ullio L. (2002) To net or not to net that is the question! But is it the answer? In: Eby P, Lunney D, Ed. *Managing the Grey-headed flying fox as a threatened species in NSW*. Mosman, NSW: Royal Zoological Society of NSW. 273–284., Vol. 32, No. 1, pp. 70-77

Upham N, Burgin C, Widness J, Liphardt S, Parker C, Becker M, Rochon I, Huckaby D. (2020). Mammal Diversity Database. Mammal Diversity Database (Version 1.2) [Data set]. *Zenodo*, doi:10.5281/zenodo.4139818

Vardon MJ, Brocklehurst PS, Woinarski JCZ, Cunningham RB, Donnelly CF and Tidemann CR. (2001). Seasonal habitat use by flying foxes, *Pteropus alecto* and *P. scapulatus* (Megachiroptera), in monsoonal Australia. *Journal of Zoology* 253, 523–535.

van der Ree R, McDonnell MJ, Temby ID, Nelson J and Whittingham E. (2006). The establishment and dynamics of a recently established urban camp of Pteropus poliocephalus outside their geographic range. *Journal of Zoology*, 268, 177–85. doi: 10.1017/S0952836901000486

Vogel G. (2003). Can great apes be saved from Ebola? *Science*, 300:1645. doi: 10.1126/science.300.5626.1645

VanDerWal J, Murphy H, Kutt A, Perkins GC, Bateman BL, Perry JJ, Reside AE. (2013). Focus on poleward shifts in species' distribution underestimates the fingerprint of climate change. *Nature Climate Change,* 3: 239–243. doi: 10.1038/nclimate1688

Walsh PD, Abernethy KA, Bermejo M, Beyers R, De Wachter PD, Akou ME, Huijbregts B, Mambounga DI, Toham AK, Kilbourne AM, Lahm SA, Latour S, Maisels F, Mbina C, Mihindou Y, Obian SN, Effa EN, Starkey MP, Telfer P, Thibault M, Tutin CEG, White LJT, Wilkie DS. (2003). Catastrophic ape decline in western equatorial Africa. *Nature*, 422:611–4. doi: 10.1038/nature01566

Walsh PD, Biek R, Real LA. (2005). Wave-like spread of Ebola Zaire. *PLoS Biology,* 3(11). doi: 10.1371/journal.pbio.0030371

Walther G, Post E, Convey P, Menzel A, Parmesan C, Beebee TCJ, Fromentin J-M, Hoegh-Guldberg O, Bairlein F. (2002). Ecological responses to recent climate change. *Nature*, 416, 389–95. doi: 10.1038/416389a

Walton Z, Samelius G, Odden M and Willebrand T. (2018). Long-distance dispersal in Red foxes, *Vulpes vulpes*, revealed by GPS tracking. *European Journal of Wildlife Research,* 64 (2018). doi: 10.1007/s10344-018-1223-9

Wang L.-F, Walker PJ, and Poon LLM. (2011). Mass extinctions, biodiversity and mitochondrial function: are bats 'special' as reservoirs for emerging viruses? *Current Opinion in Virology,* 1, 649–657. doi: 10.1016/j.coviro.2011.10.013

Warrilow D, Smith IL, Harrower B and Smith GA. (2002). Sequence analysis of an isolate from a fatal human infection of Australian bat lyssavirus. *Virology,* 297:109–119. doi:10.1006/viro.2002.1417

Webb NJ and Tidemann CR. (1995). Hybridisation between Black (*Pteropus alecto*) and Grey-headed (*P. poliocephalus*) flying foxes (Megachiroptera: Pteropodidae). *Australian Mammalogy*,19-26.

Webb NJ and Tidemann CR. (1996). Mobility of Australian flying foxes, Pteropus spp (Megachiroptera): Evidence from genetic variation. *Proceedings of the Royal Society of London Series B - Biological Sciences,* 263: 497-502.

Welbergen JA. The social organisation of the Grey-headed flying-fox, *Pteropus poliocephalus*. PhD thesis. Cambridge: University of Cambridge; 2005

Welbergen JA, Klose SM, Markus N & Eby P. (2008). Climate change and the effects of temperature extremes on Australian flying foxes. *Proceedings of the Royal Society B: Biological Sciences* 275, 419–425. doi: 10.1098/rspb.2007.1385

Welbergen JA, Meade J, Field HE, Edson D, McMicheal L, Shoo LP, Praszczalek J, Smith C, Martin JM. (2020). Extreme mobility of the world's largest flying mammals creates key challenges for management and conservation. *BMC Biol*ogy 18, 101. doi: 10.1186/s12915-020-00829-w

Westcott DA, McKeown A, Murphy HT, Fletcher CS. (2011). A monitoring method for the Grey-headed flying fox, *Pteropus poliocephalus*. CSIRO Publishing Guidelines. Available here. Last accessed on 1 December 2020.

Williams NSG, McDonnell MJ, Phelan GK, Keim LD, van der Ree R. (2006). Range expansion due to urbanization: Increased food resources attract Grey-headed Flying foxes (*Pteropus poliocephalus*) to Melbourne. *Austral Ecology*, 31: 190-198. doi: 10.1111/j.1442-9993.2006.01590.x

Williamson M, Hooper P, Selleck P, Gleeson L, Daniels P, Westbury H, Murray P. (1998). Transmission studies of Hendra virus (equine morbillivirus) in fruit bats, horses and cats. *Australian Veterinary Journal,* 76, 813-818.

Woods R. (2013). Hendra virus (HeV) and Australian Wildlife. Wildlife Health Australia. Accessed November 2020.

Yang XL, Zhang YZ, Jiang RD, Guo H, Zhang W, Li B, Wang N, Wang L, Waruhiu C, Zhou JH, Li SY, Daszak P, Wang LF, Shi ZL. (2017). Genetically diverse filoviruses in *Rousettus* and *Eonycteris* spp. bats, China, 2009 and 2015. *Emerging Infectious Diseases,* 23:482–486. doi: 10.3201/eid2303.161119

Yuan JF, Zhang YJ, Li JL, Zhang YZ, Wang LF, Shi ZL. (2012). Serological evidence of Ebolavirus infection in bats. *China Virology Journal,* 9:236. doi: 10.1186/1743-422X-9-236

Yob JM, Field HE, Rashdi AM, Morrissy C, van der Heide B, Rota P, bin Adzhar A, White J, Daniles P, Jamaluddin A, Ksiazek T. (2001). Nipah Virus Infection in bats (order Chiroptera) in Peninsular Malaysia. *Emerging Infectious Diseases,* 7(3). doi: 10.3201/eid0703.010312

Zhang C, Wang Z, Cai J, Yan X, Zhang F, Wu J, Xu L, Zhao Z, Hu T, Tu C, He B. (2020). Seroreactive Profiling of Filoviruses in Chinese Bats Reveals Extensive Infection of Diverse Viruses. *Journal of Virology,* 94 (7) e02042-19; doi: 10.1128/JVI.02042-19

Zhou P, Yang X-L, Wang X-G, Hu B, Zhang L, Zhang W, Si, H-R, Zhu, Y, Li B, Huang C-L, Chen H-D, Chen J, Luo Y, Guo H, Jiang R-D, Chen Y, Shen X-R, Wang X, Zheng X-S, Zhao K, Chen Q-J, Deng F, Liu L-L, Yan B, Zhan F-X, Wang Y-Y, Xiao G-F, Shi Z-L. (2020). A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature,* 579: 270-273. doi:10.1038/s41586-020-2012-7

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Chapter 2

Spring foraging movements of an urban population of Grey-headed flying foxes (*Pteropus poliocephalus).*

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Keywords: urban, foraging, global positioning systems, Grey-headed flying fox, Pteropus poliocephalus

2.1 Abstract

Flying foxes provide ecologically and economically important ecosystem services but extensive clearing and modification of habitat and drought combined with the planting of commercial and non-commercial trees across various landscapes, has meant flying foxes in Australia are increasingly seeking foraging resources in new areas. In 2011, Grey-headed flying foxes formed a camp in Adelaide, South Australia outside their previously recorded range. We used GPS telemetry to study the movements and foraging behaviour of this species in Adelaide in Spring (September - November) 2015. High frequency location data was used to determine the foraging range and the most frequently visited foraging sites used by each bat which were ground-truthed to identify forage plants. A total of 7,239 valid locations were collected over 170 nights from four collars. Despite being a highly mobile species, the mean core foraging range estimate was only 7.30 km^2 (range 3.3–11.2 km^2). Maximum foraging distance from the camp in the Botanic Park was 9.5 km but most foraging occurred within a 4 km radius. The most common foraging sites occurred within the residential area of Adelaide and included introduced forage plant species, Lemon-scented gum (*Corymbia citriodora*) and Port Jackson Fig (*Ficus rubiginosa*). Other observed movement activities included dipping behaviour on inland and marine waters and travel across flight paths around Adelaide airport. Our findings suggest that urban habitats in Adelaide provide sufficient foraging resources for Grey-headed flying foxes to use these areas exclusively, at least in Spring. This creates substantial opportunities for bats to interact with humans and their infrastructure.

2.2 Introduction

The distribution of Australian flying foxes (Pteropodidae, Chiroptera) is changing in response to habitat loss (Markus and Hall, 2004; McDonald-Madden et al, 2005; van der Ree et al, 2006), competition for resources (Webb and Tidemann 1995) and other global change phenomena including climate change (Parris and Hazell 2005; Kessler et al, 2018). In some instances, this has resulted in the expansion and establishment of flying fox camps in urban areas (Williams et al, 2006). Evidence from a range of urban-dwelling animals indicates that some of these urban visitors alter their ecological traits to adapt to urbanisation, including their movement and migratory behaviour (Lowry et al, 2013) and foraging preferences (Contesse et al, 2004). Flying foxes use most habitats in which suitable foraging resources are to be found and compared to natural forests, urban environments can provide increased availability and easier access of food resources (Nakamoto et al, 2012; van der Ree et al, 2006; McDonald-Madden et al, 2005). At least 20 species of bats have found useful resources in urban environments (McFarlane et al, 2015) and some urban areas may support a greater diversity of bats than forested areas (Threlfall et al, 2013).

The Grey-headed flying fox (*Pteropus poliocephalus*) is listed as Vulnerable under the *Environment Protection and Biodiversity Conservation Act 1999* (Threatened Species Scientific Committee 2001). The species is endemic to the eastern states of Australia with the majority of the population found from southeast Victoria through to Mackay (Westcott et al, 2015). More recently, they have expanded their range, as far north as Innisfail in Queensland, along the western slopes of the Great Dividing Range (Westcott et al, 2015), and to the west, as far as the study camp in Adelaide.

Grey-headed flying foxes are a generalist nectarivore and frugivore (Schmelitschek et al, 2009) and use food resources such as mangroves, coastal and montane woodlands (Woinarski et al, 2014; Westcott et al, 2015). The species is considered a sequential specialist, that is, within one area it will use a limited number of food sources hierarchically consuming a plentiful resource until it is

consumed or becomes unavailable (Parry-Jones and Augee 1991). Common food trees include the fruits of *Moraceae*, the blossom of *Myrtaceae*, *Proteaceae* and a variety of planted native and exotic trees in urban areas (Eby 1991; Parry Jones and Augee 1991; Tidemann 1999; van der Ree et al, 2006; Williams et al, 2006; Schmelitschek et al, 2009; Griffiths et al, 2020).

In 2011, Grey-headed flying foxes expanded their former range and formed a camp in Adelaide's Botanic Park, South Australia, a popular recreational site for the city's populace. Since that time, the camp has increased from an estimated 300 to 20,000 individuals (J Van Weenen 2020, pers. comm.) through breeding and seasonal immigration and is now classified as a nationally important permanent camp (Referral guideline, 2015). Adult counts tend to be highest in winter and lowest in summer (J Van Weenen 2020, pers. comm.). The increase in camp size has occurred despite seasonal emigration and large bat mortality events during extreme heat waves in the summers of 2017 and 2019. The reason for the Grey-headed flying fox range expansion into an urban environment is unknown but it has been suggested that flying foxes aggregate in urban environments to exploit greater food resources (Kessler et al, 2018). The presence of large bat camps in urban areas can also lead to animal-human conflicts. Flying foxes from this camp have caused power outages, occasional plane strikes, foraged in orchards and gardens (J Van Weenen 2020, pers. comm.) and are known to carry a variety of viruses with zoonotic potential (Boardman et al, 2020).

Telemetry studies have been undertaken on several *Pteropus* species in Australasia, Africa and Asia to investigate long distance movements, foraging patterns, food preferences, home range movements and roost selection (Tidemann and Nelson 2004; Breed at al. 2010; Roberts et al, 2012; Oleksy et al, 2015; Choden et al, 2018). Early studies used radiotracking to document long distance movements of the Grey-headed flying fox (Spencer et al, 1991) and Black flying fox (*Pteropus alecto*) (Palmer et al, 2000) on the east coast of Australia. The advent of satellite telemetry broadened our understanding of long-distance movements and the distribution of Black flying fox (Breed et al,

2010; Smith et al, 2011) and Grey-headed flying foxes (Tidemann and Nelson 2004; Roberts et al, 2012). By incorporating a duty cycle which provides more frequent fixes, telemetry can be used to analyse fine scale movements and foraging activities of flying foxes across local landscapes. These fine scale foraging movements have been investigated in Madagascan flying foxes (*Pteropus rufus*) (Oleksy et al, 2019) and Lyle's flying fox (*Pteropus lylei*) (Choden et al, 2019), however there is no report on fine-scale movements, foraging activities and ground-truthing of forage plants for flying foxes within urban landscapes in Australia.

Apart from sporadic anecdotal reports, we have limited understanding of the foraging resource use by Adelaide's Grey-headed flying foxes nor the extent of their nocturnal movements, and with it their potential interactions with the public. Here we documented the foraging movements of Grey-headed flying foxes from the Adelaide's camp using GPS telemetry. Our study objectives were to (i) characterise space use and foraging range over the greater Adelaide region, (ii) analyse foraging site use and (iii) ground-truth and identify foraged food plants. We expected that Grey-headed flying foxes would forage beyond the Adelaide boundaries to find sufficient and suitable food resources.

2.3 Materials and Methods

2.3.1 Ethics

A permit to undertake scientific research was granted by the Government of South Australia Department of Environment, Water and Natural Resources (M26371). Field procedures were approved by the University of Adelaide Animal Ethics Committee (S-2015-028).

2.3.2 Study site, animal capture and deployment of GPS tracking devices.

The population used for this study was the Grey-headed flying fox camp (Fig 1) established in Adelaide's Botanic Park, Adelaide, South Australia (-34.91588; 138.6065) in Aleppo pine (*Pinus halopensis*). Between 31 August and 3 September 2015, study animals were captured at the roost

site, using 12 or 18 m long mist nets (Ecotone, Gdynia, Poland) installed beneath the camp. Mist nets were raised 20 m above the ground before bats returned from their nightly foraging activity. As each bat became entrapped, the net was lowered, the bat carefully removed and placed securely in a pillowcase, and the net was then raised again to catch additional bats. The bagged bats were immediately relocated to the Animal Health Department of the adjacent Adelaide Zoo.

We attached prototype CSIRO Camazotz data loggers (Jurdak et al, 2013) to five individuals using *c*. 2-cm-wide neoprene collars with a kangaroo-leather lining. All selected bats were free from any clinical signs of disease or abnormalities and of sufficient size that the combined weight of transmitter and collar (23g) was <3% of bodyweight (Bander and Cochran. 1991). Collars were fitted to bats under general anaesthesia (Isoflurane, Laser Animal Health) following the protocol described by Jonsson et al, (2004). The collars were closed with superglue and the join sutured using synthetic absorbable suture. This served as a weak link, allowing for eventual shedding of the collar and tracking device without further handling or intervention. Each device contained a GPS module, a temperature and air pressure logger, audio recorder and inertial units to modulate recording when bats were stationary for long periods (Jurdak et al, 2013). Tracking devices were powered by a solar panel affixed to the exterior dorsal surface for recharging batteries of 300 milliamp-hour capacity, and an antenna projecting approximately 7cm dorsally and caudally to transmit data via short range UHF radio waves.

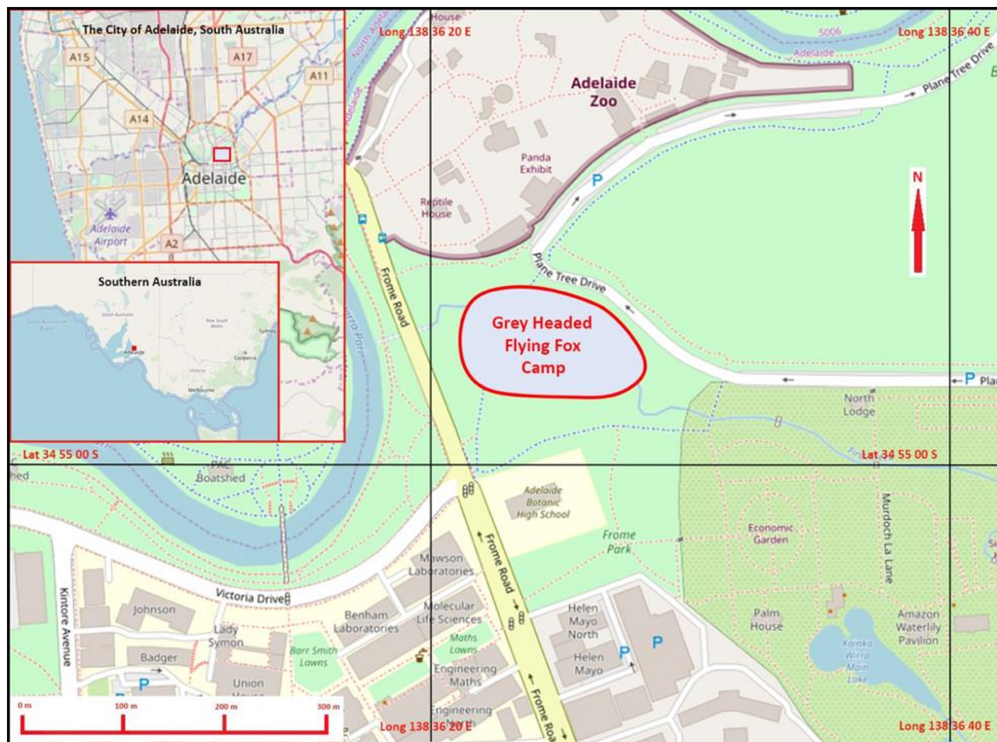

Fig 2.1: Location of the Grey-headed flying fox (*Pteropus poliocephalus*) camp in Adelaide's Botanic Park showing camp extent (red line) and proximity to Adelaide Zoo where bats were tagged. Insets illustrate central Adelaide and Southern Australia to show geographical relationships. Geodata from OpenStreetMap was downloaded via the Maperitive application and the map was rendered with further information supplied by the author.

2.3.3 Data acquisition and management

Each GPS device recorded an individual's three-dimensional position at one-second intervals unless battery life was low (<50%) when the units switched to a 10-minute or 1-hour recording interval. All times are Australian Central Standard Time and take no account of daylight-saving time. In addition to time and geolocation (longitude, latitude), each device records altitude (m) above mean sea level (AMSL), speed (m/s), number of satellites per fix and 'position dilution of precision' (PDOP). PDOP is a measure of location precision and is determined by the position of satellites in relation to the tracking device and associated imprecision in any of the four dimensions measured: time and three dimensions in space (Misra et al, 1999). Data was stored on the devices and downloaded by short-

range radio transmission daily when bats moved to within approximately 300m radius of the 3G modem base station receiver in the camp (Jurdak et al, 2013). All fixes in the dataset were managed in Movebank (Kranstauber et al, 2011). We thinned the dataset to one fix per minute and, in addition, we removed all locations between sunrise and sunset, when bats were roosting in the camp, using the *crespuscule* function in the *maptools* package (v. 0.9.5, Lewin-Koh 2011) in R (v. 3.6.2, R Core Team 2019).

2.3.4 Foraging range estimation

In ecology, kernel density estimation (KDE) is a widely used probabilistic method of home-range estimation that assumes data are independent and identically distributed. The high frequency of location fixes (every minute) in our study meant that any location fix was likely correlated with the previous or subsequent fix as individuals repeat behaviours or maintain directional movement. We therefore used a method that explicitly incorporates this autocorrelation into the estimation process, autocorrelated kernel density estimation (AKDE; Fleming et al, 2015) to estimate foraging ranges for each individual. Relocation data are ordered in time and can be modelled as a continuous-time stochastic process, and for finely sampled data, the data will tend to exhibit positional and velocity autocorrelation (Calabrese et al, 2016). AKDE is an efficient nonparametric estimator that produces more accurate measurements of space use than other estimators of home-range (Noonan et al, 2019). We used the *ctmm.select* function in R package to examine candidate models using maximum likelihood (Fleming et al, 2014) and selected the best model based on the lowest Akaike information criterion (AIC; Akaike 1973; Akaike 1974). In all cases, the best model for individuals was the anisotropic Ornstein-Uhlenbeck F (OUF) process model for individuals that display limited space use and correlated velocities (Calabrese et al, 2016). We then calculated the weighted utilisation distribution using the *akde* function for both core area and extended foraging range area.

2.3.5 Foraging sites and visitation

We used the *recurse* package (v. 1.1.0; Bracis et al, 2018) in R (v. 3.6.2; R Core Team 2019) to determine the location and frequency of visits to foraging sites. The *recurse* package counts the number of trajectory segments of the movement paths of individuals that intersect a circle specified by a radius set at 25 m around GPS fixes. It then counts the number of trajectory segments of the movement paths of one or many individuals that intersect the circle. Each such intersection was classified as one visit. The package used linear interpolation to estimate the entrance and exit times and calculated visit duration and time since previous visit. We used a frequency histogram to identify a foraging site visitation threshold of greater than 20 visits per location and selected the six most frequented foraging sites for each individual bat over the study period to ground truth what they had been eating. Further, we compared the visit frequency to each site, duration of each visitation and duration by week of the year to assess foraging site usage over time.

2.3.6 Identification of foraging plants

The most frequently visited GPS fixes selected above were ground-truthed (including 25 m radius around the fix) to identify foraging plants. Photographs were taken of trees of interest, and buds, flowers or fruiting bodies and leaf-branch structure were sampled for identification. These samples were identified, where possible to species, using expertise at the State Herbarium of South Australia and appropriate keys and identification guides. Confirmed (investigated) feeding sites were classified as park, street or private land types. “Park” was defined as a vegetated public space, sporting field, school, park and foreshore; “street” was defined as residential road frontage, curb-side, roadside footpath or median-strips and railway or highway screens, and “private land” was defined as privately owned vegetation in business premises, domestic gardens, rear yards or restricted access areas including, for example, the private off-road car park of a housing community.

2.4 Results

2.4.1 GPS tracker performance

Five adult males had tracking devices attached of which only four returned data (Table 2.1). Our GPS devices transmitted for 5-62 nights and a total of 7,239 valid locations were collected over 170 nights from the four collars (Table 2.1). These fixes included some from within the camp itself when the individual departed after sunset or returned to camp before sunrise. The proportion of valid data (i.e. data with an actual geographic location) was 99.8%. Mean PDOP across all points was 3.06, and all were within the recommended range of 2 – 5 for reliable navigation.

Table 2.1: Morphometrics, GPS collar deployment and thinned fixes and foraging distances of four adult male Grey-headed flying foxes from the Adelaide camp between 31 August 2015 and 2 November 2015. FAL = forearm length. N/A = not applicable.

Bat ID#	Weight (g)	FAL (mm)	Tagging date	First record date	Last record date	Tracking night count	Location fix count	Night time location fix count
403	883	169	03/09/15	03/09/15	21/10/15	48	3,072	2,492
657	854	166	31/08/15	31/08/15	24/10/15	55	3,684	2,901
588	846	156	01/09/15	01/09/15	02/11/15	62	2,254	1,790
684	944	173	03/09/15	03/09/15	09/09/15	5	68	46
575	851	172	31/08/15	N/A	N/A	N/A	N/A	N/A
Total						**170**	**9,078**	**7,239**

2.4.2 Individual bat movements

Each individual bat had preferred and distinct foraging pathways that included several foraging sites that they revisited multiple times (Fig 2.2). Two individuals appear to regularly follow major geographic landmarks, being Port Road (Bat #403) and the River Torrens (Bat #588), while the third followed a smaller drainage line to the foot of the Adelaide Hills (Bat #657) (Fig 2). Bat #403 ranged

mostly to the west of the camp in the Botanic Gardens with outward and return flights following the course of the River Torrens and often diverged north westerly to repeatedly visit the same foraging sites in the western suburbs. This routine remained throughout the tracking period from early September to late October. Bat #403 made two notable extensions to its regular route. One atypical flight took a path 9.6 km to the south flying across Adelaide International Airport (at ~40 m above mean sea level at ~22h10; 19 September 2015) and then a 10 km loop out to sea. On several occasions, bat #403 was recorded on the water surface of the River Torrens and made short excursions onto the sea at Henley Beach, approximately 6 km from its usual foraging sites.

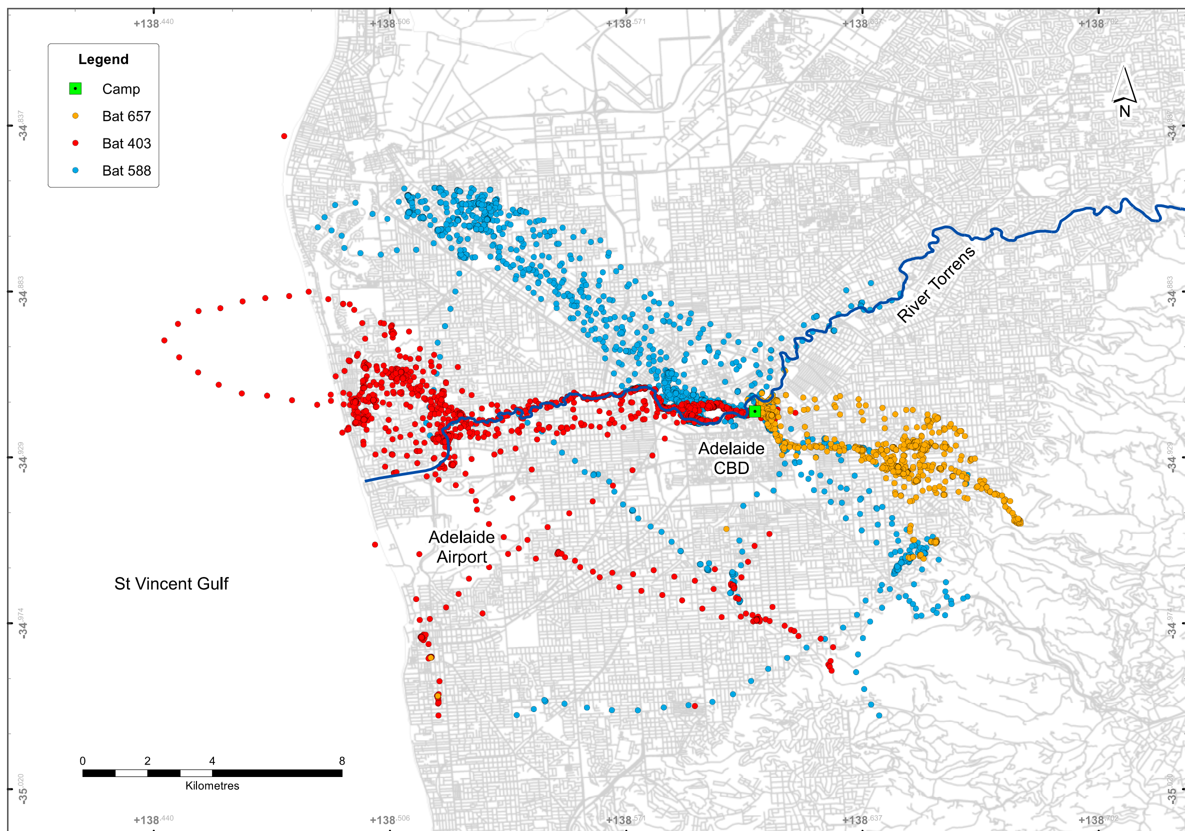

Fig 2.2: Flight paths of three Grey-headed flying foxes from the Adelaide camp between 31 August 2015 and 2 November 2015. Each dot depicts a location at least 1 minute from the previous location.

Bat #657 ranged to the east of the city repeatedly following the course of First Creek (-34.9295^0 S; 138.6443^0 E) to the eastern suburbs, with various short extensions of less than 2 km from its regular path. On two occasions it extended its route to a quarry dam at Slapes Gully (34.9469^0 S; 138.6803^0 E) and is recorded close to the estimated water surface height.

Bat #588 ranged mostly to the north-west of the camp, travelling out and back along a busy tree lined thoroughfare corresponding to Port Rd and the Port Adelaide railway line (-34.8783^0 S; 138.5321^0 E). This individual repeatedly visited selected trees in residential suburbs. Two atypical extensions to its usual course were made on two consecutive nights, one to the south of Adelaide and southeast to Brown Hill Creek (-34.9859^0 S; 138.6512^0 E), and one west along the River Torrens. The first extension was made on the same night that Bat #657 travelled south. Bat #588 also visited wetlands at St. Clair (-34.8680^0 S; 138.5322^0 E) on six occasions. Bat #684 returned only limited data at 10-minute intervals for 5 days (suggesting that battery levels were persistently low) has been excluded from Figure 2.2 because it showed limited linear data only. It ranged to the north-east following the course of the River Torrens, a vegetated and landscaped park area on both banks. It took a similar route for all recorded flight periods and foraged along the River Torrens and adjacent suburbs either side of the river. However, there was insufficient data to further analyse foraging site range or visitation.

2.4.3 Core and extended foraging-range and forage site visitation patterns

The foraging range (weighted utilisation distribution) from the camp site varied between individuals. Of the three individuals that provided sufficient data, the mean core foraging area (AKDE50), the area used for 50% of the foraging time, was 7.30 km^2 (range 3.3–11.2 km^2) (Table 2.2; Fig 2.3). The mean extended foraging range (AKDE95), was 45.0 km^2 (range 1.78–62.2 km^2) (Table 2.2; Fig 2.3). Both of these areas included the camp site, but daytime locations were not used to calculate the utilisation distribution. The frequency and pattern of visitation is illustrated in Figs 2.4, 2.5 and 2.6

for bat #403, #657 and #588, respectively. Overall, 15 frequently visited foraging sites were identified (>20 visits over the period of observations). Bats #403, #657 and #588 had 5, 6 and 5 frequently visited foraging sites, respectively. The most commonly visited foraging sites were on streets (7 of 15; 47%) and foraging plants were either not native to South Australia or exotic.

One site in close proximity to the camp in the Botanic Gardens (-34.9169^0 S; 138.6118^0 E) was used by two of the tracked bats, #403 and #657. In weeks 37–42, bat #403 often visited this site immediately after leaving the camp and revisited again before returning to the camp, suggesting a reliable food resource during that period (Fig 2.7). Productive foraging sites were visited repeatedly. For example, bat #403 spent 42 hours in total at site 3 over four weeks (36-39) feeding on a Lemon-scented gum (*Corymbia citriodora*) (Fig. 2.7). Visitation declined thereafter. Similarly, bat# 657 made visits to site 4, European olive (*Olea europaea*), during weeks 37–40 (Fig. 2.8) spending over 22 hours foraging in total at this site. This bat also foraged on Queensland box (*Lophostermon confertus)* flowers at site 6, close to site 4, during weeks 37-41 for approximately 31 hours overall. In contrast, bat #588 only visited a single site (site 2) during weeks 36–39, to forage on Port Jackson fig (*Ficus rubiginosa*) for ~41hours total (Fig. 2.9). Following foraging, all three bats regularly returned to the camp up to 2 hours before sunrise.

Table 2.2: Utilisation distribution (km^2) with confidence intervals using weighted autocorrelated kernel density estimates (AKDE) for the foraging range of three Grey-headed flying foxes from the Adelaide camp between 31 August 2015 and 2 November 2015 with meaningful data.

Bat ID #	Core Utilisation distribution defined by AKDE 50 (km^2) (95% CI)	Extended Utilisation distribution defined by AKDE 95 (km^2) (95% CI)
403	11.2 (9.8-12.6)	62.2 (57.1-73.8)
657	3.3 (2.9-3.6)	17.8 (15.8-19.9)
588	7.4 (6.5-8.3)	55.0 (48.7-61.7)

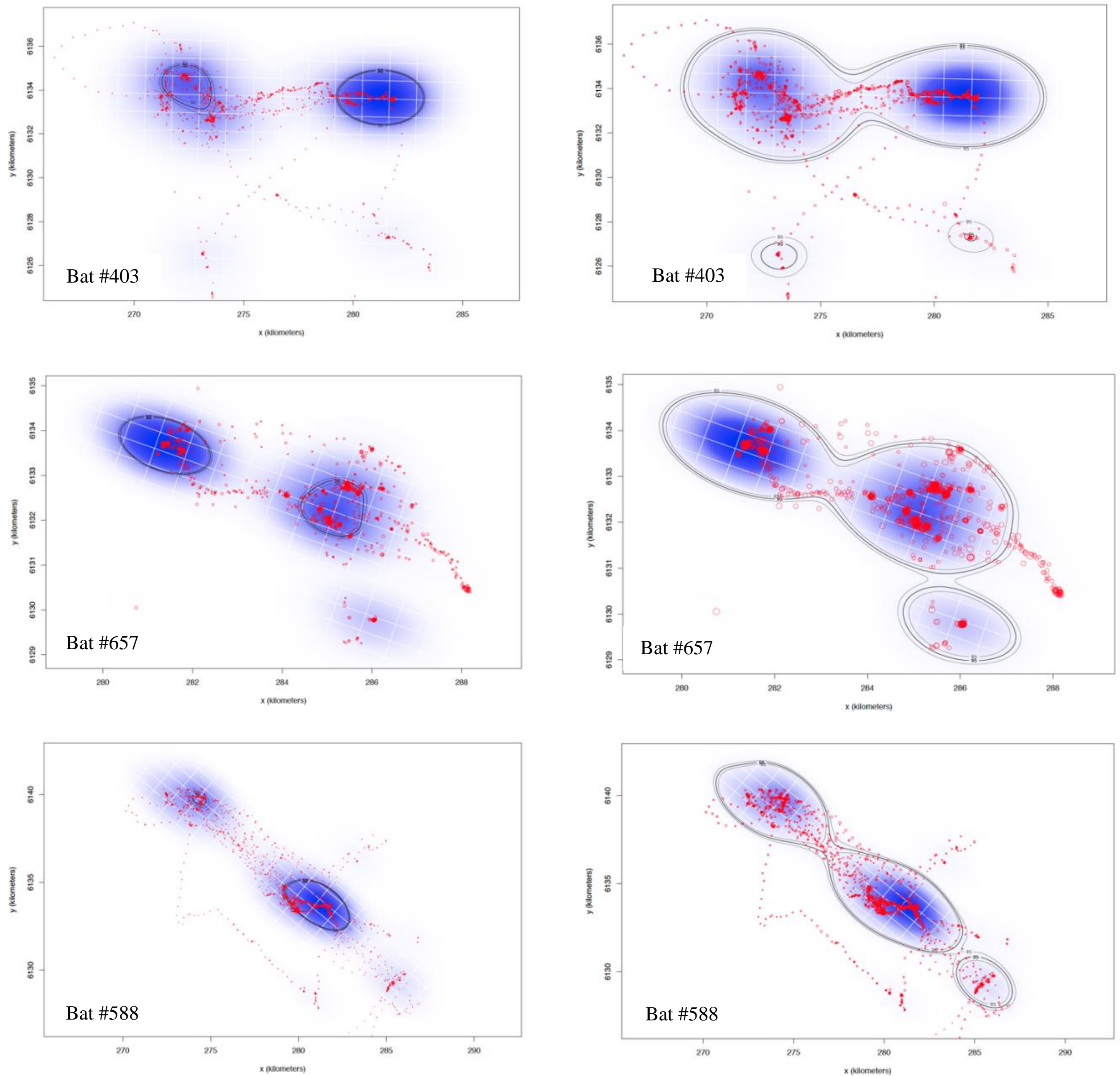

Fig 2.3: Utilisation distribution (blue) and core and extended home-range estimates with confidence intervals for the period between 31 August 2015 and 2 November 2015. Core home-range (AKDE50) (left) and extended home-range (AKDE95) (right). Lighter contours represent confidence intervals and the grid lines provide a scale in kilometres.

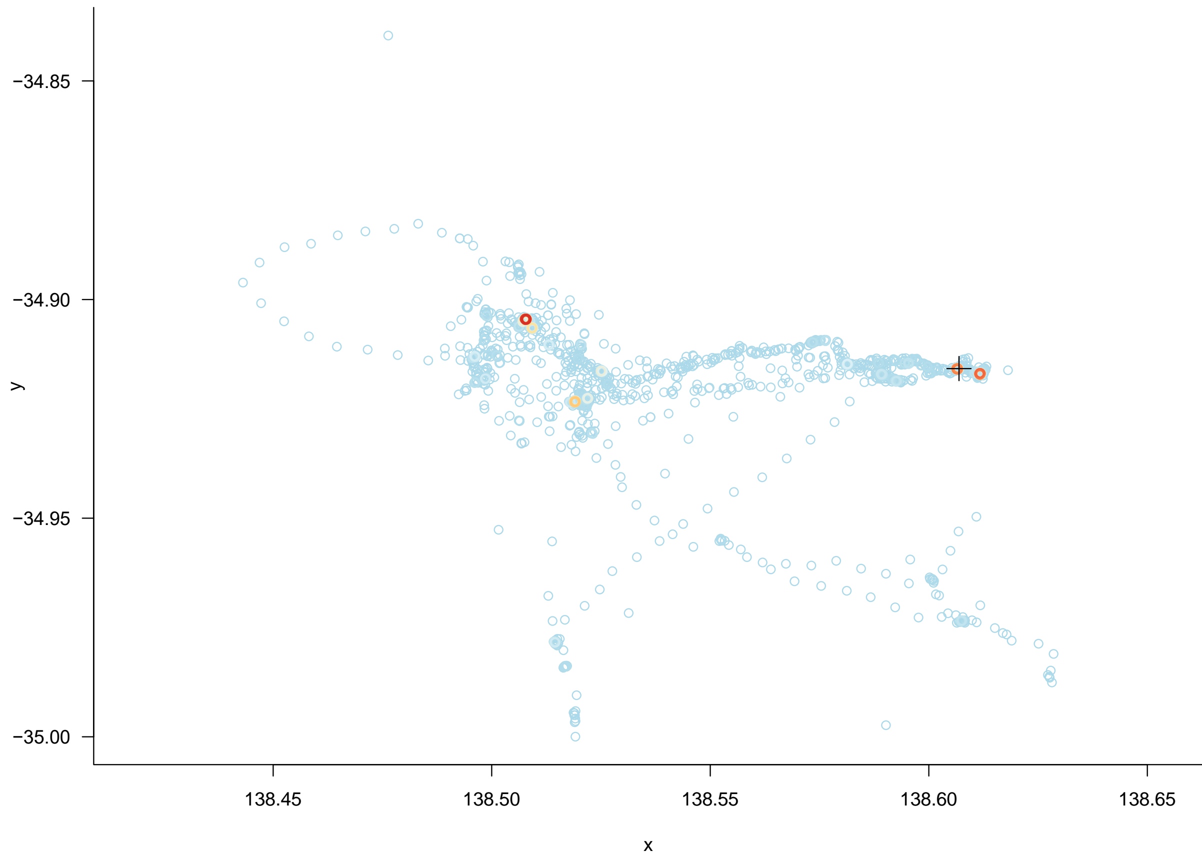

Fig 2.4: Map of pattern of revisitation for bat #403, between 3rd September 2015 to 21st October 2015 (weeks 36-42 of 2015) from the Adelaide camp. Circles mark locations: the warmer the colour of the circle is, the higher frequency of visitation. See Table 3 for GPS locations of the most visited foraging sites. The camp is represented by the cross.

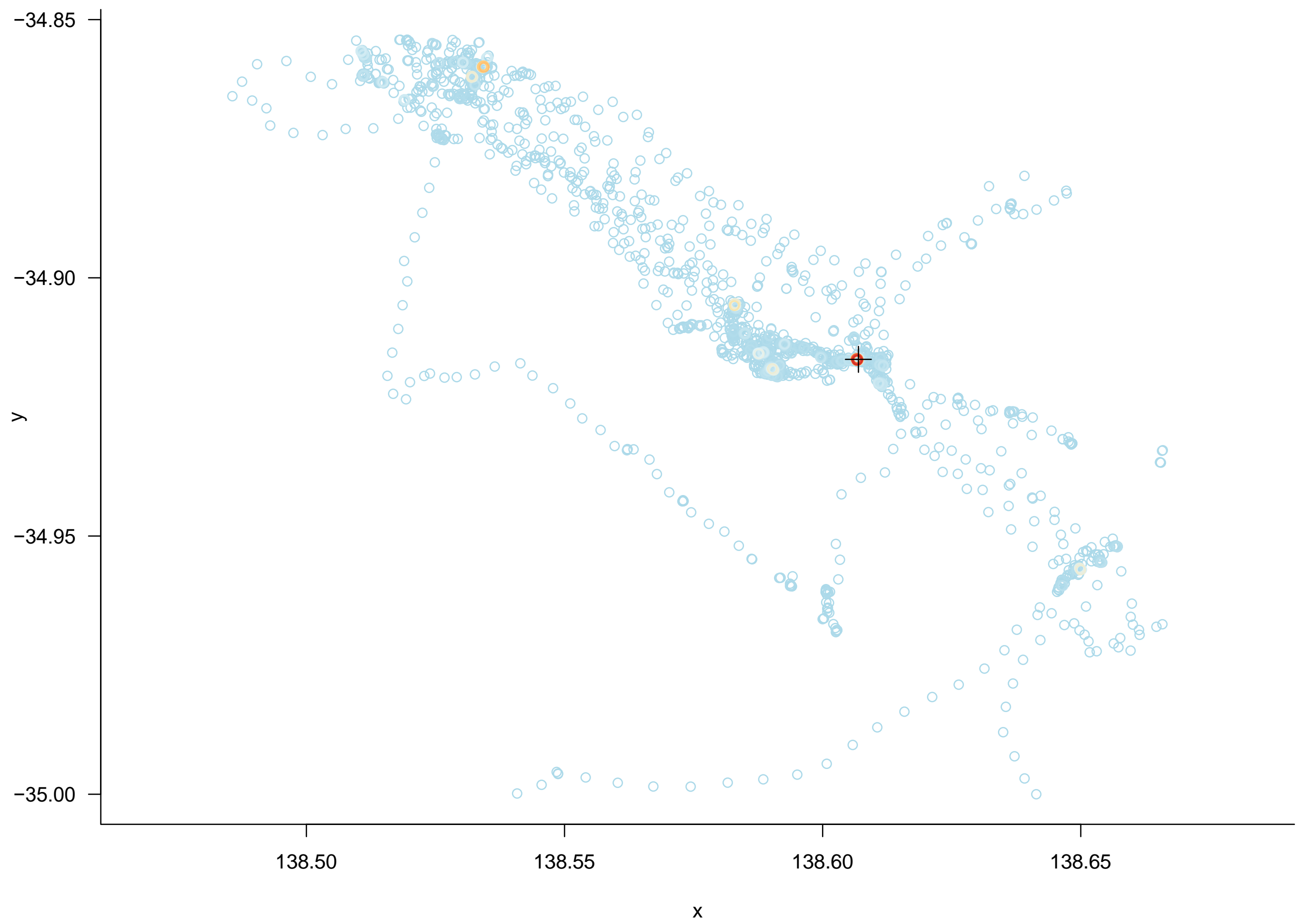

Fig 2.5: Map of pattern of revisitation for bat #657 between 31st August 2015 to 24th October 2015 (weeks 36-42 of 2015) from the Adelaide camp. Circles mark locations: the warmer the colour of the circle is, the higher the frequency of visitation. See Table 3 for GPS locations of the most visited foraging sites. The camp is represented by the cross.

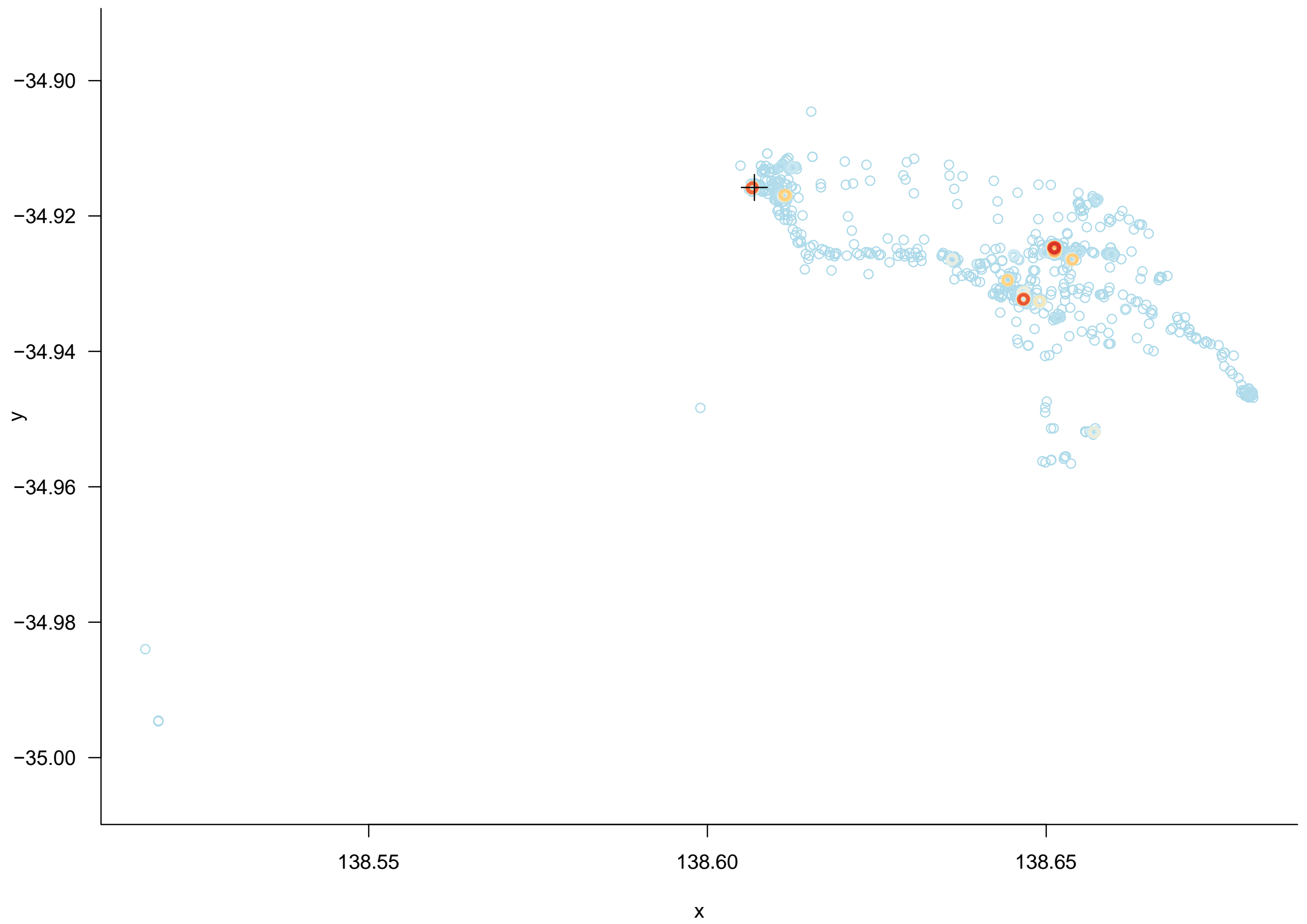

Fig 2.6: Map of pattern of revisitation for bat #588 between 1st September 2015 to 2nd November 2015 (weeks 35-44 of 2015) from the Adelaide camp. Circles mark locations: the warmer the colour of the circle is, the higher the frequency of visitation. See Table 3 for GPS locations of the most visited foraging sites. The camp is represented by the cross.

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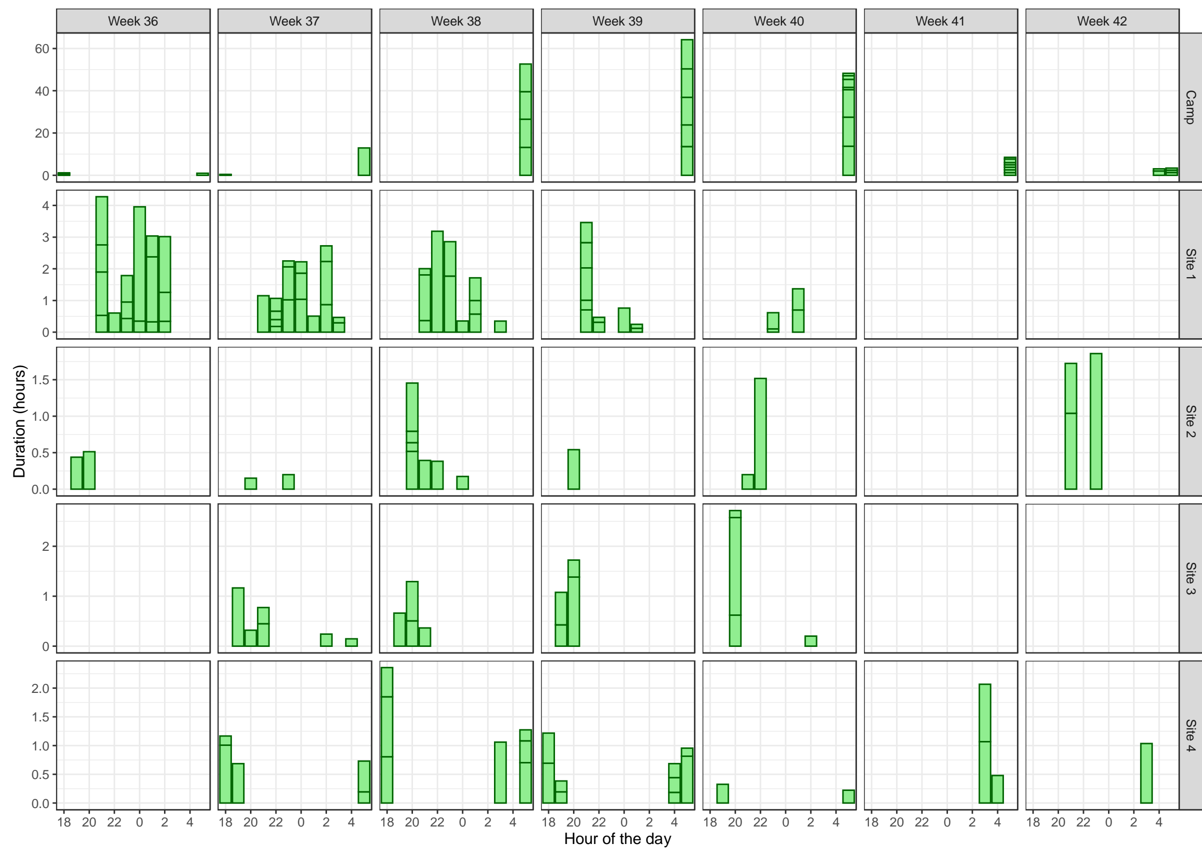

Fig 2.7: Foraging activity histogram for Grey-headed flying fox, bat #403 between 3rd September 2015 to 21st October 2015 (weeks 36-42 of 2015) including for comparison camp location and most frequently visited sites including frequency, timing and duration of visitation.

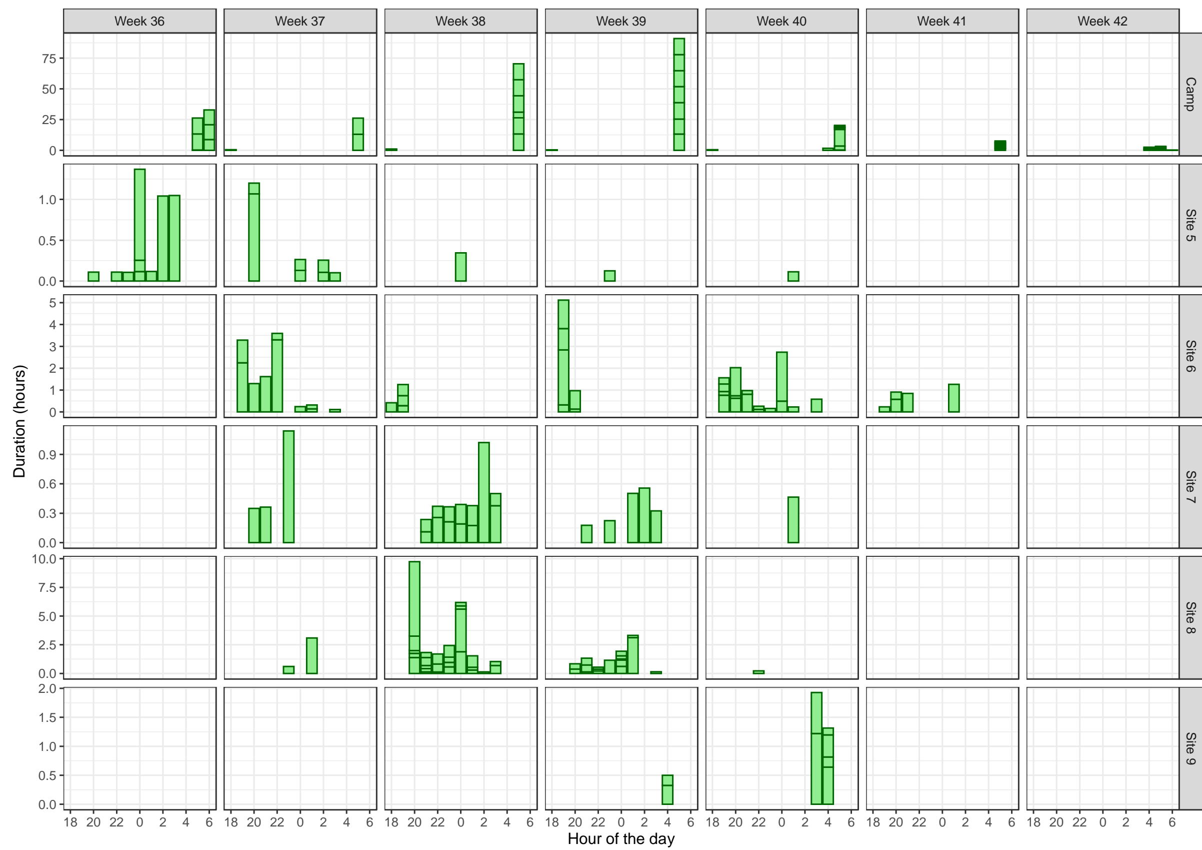

Fig 2.8: Foraging activity histogram for Grey-headed flying fox, bat #657 between 31st August 2015 to 24th October 2015 (weeks 36-42 of 2015) including for comparison, camp location (site 1) and most commonly visited sites including frequency, timing and duration of visitation.

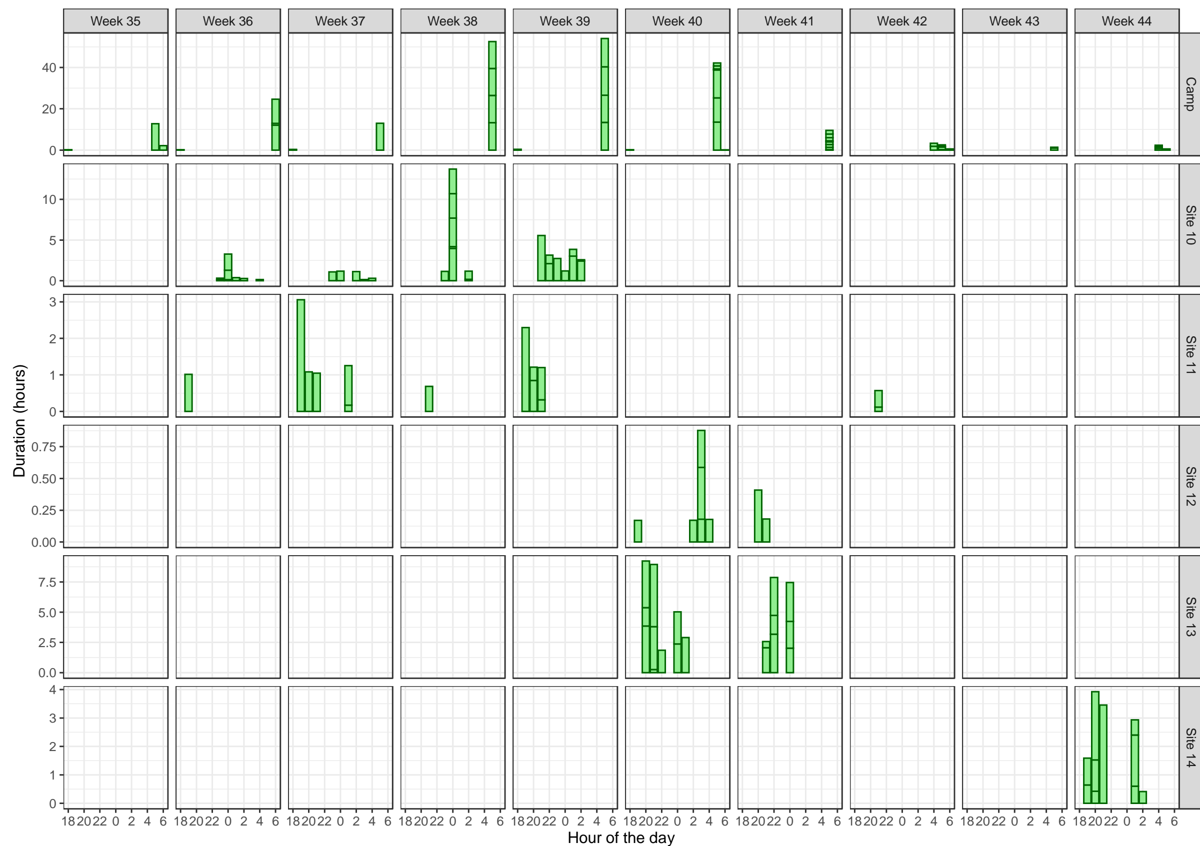

Fig 2.9: Foraging activity histogram for Grey-headed flying fox, bat #588 between 1st September 2015 to 2nd November 2015 (weeks 35-44 of 2015) including for comparison, camp location (site 1) and most commonly visited sites including frequency, timing and duration of visitation.

2.4.4 Identification of foraging plants

Fourteen plants used by Grey-headed flying foxes were identified at the most visited foraging sites (Table 2.3). At seven foraging sites, there were more than one plant species. Common Spring forage plants included the nectar of the Lemon-scented gum (3 sites), fruit of the Port Jackson Fig (3 sites) and the nectar of the Yellow box (*Eucalyptus melliodora)* (2 sites). Other forage species identified at these sites, including the Flooded gum (*E. grandis)* and Sugar gum (*E. cladocalyx)*, do not flower during Spring and were therefore unlikely to be a food source at the time of our study. Of the species identified at the foraging sites only River red gum (*E. camaldulensis)* and Sugar gum are native to South Australia and neither flower in Spring. All other species identified are either not

native to South Australia (10 species) or are exotic to Australia (2 species) and were introduced to the Adelaide region following European settlement. Plants at two locations (once each for bat #657 and bat #588 could not be identified due to access restrictions.

Table 2.3: Ground-truthed plants associated with the most frequently visited foraging sites of Grey-headed flying foxes in Adelaide between 31 August 2015 and 2 November 2015 inferred from GPS. Only trees accessed and identified are listed. Land type categories are determined by the location of the base of the tree. “Park” includes public spaces, sporting fields, schools, parks and foreshore. “Street” includes residential curb-side streets, road frontage, median strip, or transport corridor screens. “Private” includes privately owned trees in domestic gardens, business premises or restricted-access areas. Bold type indicates plants known to flower or grow fruit during Spring. * denotes exotic to Australia. ** denotes this data could not be captured in foraging activity histograms (Figs 5 and 6). N/A denotes access to identify trees was not possible.

Bat ID#	Site ID	Longitude	Latitude	Type	Forage Plants			
403	1	-34.9045	138.5077	Private	***Corymbia citriodora***	*Eucalyptus sideroxylon*		
403	2	-34.9065	138.5092	Private	***Callistemon sp***			
403	3	-34.9233	138.5190	Street	***Ficus rubiginosa***	*Eucalyptus camaldulensis*		
403	4	-34.9169	138.6118	Park	***Ficus macrophylla***	***Corymbia maculata***	*Podocarpus elatus*	*Eucalyptus grandis*
403	**	-34.9210	138.5228	Street	****Phoenix canariensis***			
657	**	-34.9169	138.6118	Park	***Ficus macrophylla***	***Corymbia maculata***	*Podocarpus elatus*	*Eucalyptus grandis*
657	5	-34.9338	138.6496	Private	N/A			
657	6	-34.9295	138.6443	Street	****Olea europaea***	*Eucalyptus sideroxylon*		
657	7	-34.9323	138.6466	Private	***Eucalyptus melliodora***			
657	8	-34.9264	138.6539	Street	***Lophostemon confertus***	*Eucalyptus sideroxylon*		
657	9	-34.9247	138.6512	Street	***Corymbia citriodora***			
588	10	-34.8591	138.5343	Park	***Ficus rubiginosa***			

588	11	-34.8611	138.5321	Street	N/A		
588	12	-34.9176	138.5902	Park	***Eucalyptus melliodora***	*Eucalyptus camaldulensis*	*Eucalyptus cladocalyx*
588	13	-34.9052	138.5830	Street	***Corymbia citriodora***	***Eucalyptus salmonophloia***	
588	14	-34.9564	138.6499	Park	***Ficus rubiginosa***		

2.5 Discussion

Our study provides insights into the fine scale movements of Grey-headed flying foxes in an urban environment in Australia. Our data show that, during Spring 2015, the four tracked Grey-headed flying foxes foraged entirely within the urban area of Adelaide on tree species either non-native to South Australia or exotic trees. A similar preference for urban plantings of forage trees was observed in Cambodia in the only other study to examine fine-scale movements and foraging preferences of a large flying fox (Choden et al, 2019). Consistent with other studies across Australia, Grey-headed flying foxes repeatedly returned to the same foraging sites at similar times over several nights and weeks often for extended periods of time. Recorded distances covered nightly were consistent with those reported for individuals in Queensland and New South Wales which foraged within a 20 km radius of the camp (Eby 1991; Tidemann 1999). However, in these studies the primary foraging sites were remnant native forest patches areas within largely agricultural landscapes. We found that all collared bats had small core home ranges, with the most commonly visited foraging sites within 9.5 km of the roost camp and most of regular foraging sites occurring within a 4 km radius of the camp. This relatively small foraging range suggests that food resources were plentiful for the Grey-headed flying fox population, estimated to be approximately 3000 (J Van Weenen 2020, pers. comm.). Bats often returned to the camp before sunrise suggesting they found sufficient food resources in the time that they were foraging. Consistently between 2015 and 2018, the body condition of flying foxes in this camp in Adelaide was better in spring than in summer (Boardman et al, 2020) which is opposite to the findings of Grey-headed flying foxes in Queensland and New South Wales (L Collins 2019, pers. comm). This further indicates that food resources are relatively plentiful in residential Adelaide in spring.

Optimising foraging activities, and ultimately survival, is contingent on an individual's ability to locate and consume food at a rate sufficient to maintain physiological functions and improve fitness (Krebs 2009). Any change in the environment that allows improved foraging efficiency, such as

expansion of human-dominated urban development, is an opportunity to be exploited. Frugivores and nectarivores like flying foxes and birds can benefit from increased availability of resources in urban areas (Nakamoto et al, 2007; Nakamoto et al, 2012; Wood and Esaian 2020). Food predictability in urban landscapes shapes foraging patterns (Egert-Berg et al, 2018), and the predictability of the location of nectar and fruit resources emphasises the role played by spatial memory for guidance (Genzel et al 2018) and allows for fidelity to the same foraging sites over multiple nights (Egert-Berg et al, 2018) or weeks (Korine et al, 1999). This is reflected in frequently used flight routes from roost to foraging sites (Genzel et al, 2018) which was noted in our study.

Our results suggest that Grey-headed flying foxes in Adelaide were feeding on species that are not native to South Australia but rather were feeding on the same species found in their previous known geographic range (Queensland and New South Wales) or on exotic species. This reflects studies of the Melbourne population of Grey-headed flying foxes where approximately 40% of feeding observations were in parks on exotic plant genera (McDonald-Madden et al, 2005). We found the most commonly visited foraging sites were on streets where 100% of the foraging plants were not native to South Australia. Of the 201 species of recorded foraging plants for Grey-headed flying foxes (Williams et al, 2006), 133 have been planted within Adelaide, including 39 species, exotic to Australia (M O'Leary 2020, pers. comm.). Only 16 species recorded in the diet of Grey-headed flying foxes are found naturally in Adelaide and these do not flower or fruit in Spring when this study was conducted. Hence the dependence of this population on introduced and exotic tree species during this study. By comparison in Victoria, Australia, Williams et al, (2006) found that 87 plant species that provide food for Grey-headed flying foxes have been planted in Melbourne, as compared to only 13 naturally occurring species.

The two most common tree species frequented by Grey-headed flying foxes in this study were Port Jackson Fig and Lemon-scented gum flower each year (Table 2.3). The Port Jackson fig, native to the

eastern coastal forests of Queensland and northern New South Wales (Boland et al, 2006), is frequently used as a food source by Grey-headed flying foxes elsewhere (Williams et al, 2006; Schmelitschek et al, 2009). Foraging sites of this species in Adelaide occurred in parks and a school ground and were visited repeatedly. The natural distribution of the Lemon-scented gum is eastern Queensland (Brooker and Kleinig 2012) and is often planted in Adelaide as a municipal street tree. This species flowers from June to November (Boland et al, 2006) each year. Lesser used species such as the Yellow box occur naturally along the east coast of Australia from southern Queensland to northern Victoria (Brooker and Kleinig 2012). Yellow box is a common component of Grey-headed flying fox diet (Williams et al, 2006) and flowers from September to December (Boland et al, 2006), making it available as a foraging source in early spring in Adelaide. GPS data revealed individuals visited known water sources in the Adelaide Botanic Gardens and wide areas of the River Torrens close to the camp, as well as suburban drainage ponds, an artificial quarry dam and the sea. We presumed that bats were dipping or drinking on these occasions but the case of the movement over the sea could be considered as an aborted dispersion attempt (A McKeown 2019, pers. comm.)

Conclusion

Grey-headed flying foxes ranged and foraged on introduced plants across the Adelaide metropolitan area during spring 2015. The planting of street trees, in particular, provided foraging resources for the tracked individuals and likely for the camp as a whole. The establishment of urban camps of the Grey-headed flying fox raises numerous questions about their adaptive ecology and their potential to interact with human populations—most notably, during heat stress events or when individual bats stray into high-risk environments such as the flight paths around Adelaide airport. Further and extended satellite or GPS telemetry investigations would provide further insights into the fine scale movement ecology of this nationally important camp of flying foxes.

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Data availability

The datasets generated analysed during the current study are available in the Movebank Data Repository, https://doi.org/10.5441/001/1.5bd6pq55 (Boardman and Roshier 2020)

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2.7 References

Akaike H. (1973). Information theory and an extension of the maximum likelihood principle, *in* B. N. Petrov and F. Csaki, editors. *Proceedings of the 2nd International Symposium on Information Theory*. Akademiai Kiado, Budapest: 267–281.

Akaike H. (1974). A new look at the statistical model identification. *IEEE Transactions on Automatic Control*, 19: 716–723.

Bander R and Cochran W. (1991). Radio-location telemetry, in *Wildlife Management Techniques*. Washington DC, USA: Wildlife Society.

Boardman WSJ, Baker ML, Boyd V, Crameri G, Peck GR, Reardon T, Smith IG, Caraguel CGB, Prowse TAA. (2020). Seroprevalence of three paramyxoviruses; Hendra virus, Tioman virus, Cedar virus and a rhabdovirus, Australian bat lyssavirus, in a range expanding fruit bat, the Grey-headed flying fox (*Pteropus poliocephalus*). *PLOS One*. doi: 10.1371/journal.pone.0232339

Boardman WSJ and Roshier D. (2020). Data from: Spring foraging movements of an urban population of Grey-headed flying foxes (*Pteropus poliocephalus*), *Movebank Data Repository*. doi: 10.5441/001/1.5bd6pq55

Boland DJ, Brooker MIH, Chippendale GM, Hall N, Hyland BPM, Johnston RD, Kleinig DA, McDonald MW, Turner JD. (2006). *Forest Trees of Australia*, 5th edn. Canberra, Australia: CSIRO Publishing.

Bracis C, Bildstein KL and Mueller T. (2018) Revisitation Analysis uncovers spatio-temporal patterns in animal movement data. *Ecography*, 41: 1801-1811. doi: 10.1111/ecog.03618

Breed AC , Field HE, Smith CS, Edmonston J, Meers J. (2010). Bats Without Borders: Long Distance Movements and Implications for Disease Risk Management. *EcoHealth*, 7: 204 – 212. doi: 10.1007/s10393-010-0332-z

Brooker I and Kleinig D. (2012). *Eucalyptus. An Illustrated Guide to Identification*, Sydney, Australia: Reed New Holland.

Calabrese JM, Fleming CH and Gurarie E. (2016). ctmm: An R package for analyzing animal relocation data as a continuous-time stochastic process. *Methods in Ecology and Evolution,* 7: 1124–1132. doi: 10.1111/2041-210X.12559

Choden K, Ravon S, Epstein JH, Hoem T. Furey N, Gely M, Jolivot A, Hul V, Neung C, Tran A, Cappelle J. (2019). *Pteropus lylei* primarily forages in residential areas in Kandal, Cambodia. *Ecology and Evolution*, 9: 4181–91. doi: 10.1002/ece3.5046

Contesse P, Hegglin D, Gloor S, Bontadina F, Deplazes P. (2004). The diet of urban foxes (*Vulpes vulpes*) and the availability of anthropogenic food in the city of Zurich, Switzerland. *Mammalian Biology*, 69*:* 81–95. doi: 10.1078/1616-5047-00123

Eby P. (1991). Seasonal movements of Grey-headed Flying foxes, *Pteropus poliocephalus* (Chiroptera: Pteropodidae), from two maternity camps in northern New South Wales. *Wildlife Research,* 18: 547–59. doi: 10.1071/WR9910547

Egert-Berg K , Hurme ER, Greif S, Goldstein A, Harten L, Gerrardo Herrera M L, Flores-Martinex JJ, Valdes T, Johnston DS, Eitan O, Borissov I, Shipley JR, Medellin RA, Wilkinson GS, Goerlitz HR, Yovel Y.(2018). Resource ephemerality drives social foraging in bats. *Current Biology*, 28: 3667–73. doi 10.1016/j.cub.2018.09.064

Fleming CH, Calabrese JM, Mueller T, Olson KA, Lemigruber P, Fagan WF, Boler B, Bronstein JL. (2014). From fine-scale foraging to home ranges: A semi-variance approach to identifying movement modes across spatiotemporal scales. *The American Naturalist*, 183: doi: 10.1086/675504

Fleming CH, Fagan WF, Mueller T, Olson KA, Lemigruber P, Calabrese JM,. (2015). Rigorous home range estimation with movement data: a new autocorrelated kernel density estimator. *Ecology,* 96:1182–1188. doi: 10.1890/14-2010.1

Genzel D, Yovel Y and Yartsev MM. (2018). Neuroethology of bat navigation. *Current Biology,* 28: 997–1004. doi: 10.1016/j.cub.2018.04.056

Griffith P, Parry-Jones K and Cunningham AA. (2020). Dietary partitioning in newly sympatric urban flying foxes (*Pteropus poliocephalus* and *Pteropus alecto*). *Australian Mammalogy* 42, 361-366. doi: 10.1071/AM19047

Jonsson NN, Johnston SD, Field H, de Jong C, Smith C. (2004). Field anaesthesia of three Australian species of flying fox. *Veterinary Record,* 154: 664. doi: 10.1136/vr.154.21.664

Jurdak R, Sommer P, Kusy B, Kottege N, Crossman C, McKeown A, Westcott D. (2013). Camazotz: Multimodal Activity-based GPS Sampling. In Proceedings of *The International Conference on Information Processing in Sensor Networks,* 67-78. doi: 10.1145/2461381.2461393

Kessler MK, Becker DJ, Peel AJ, Justice NV, Lunn T, Crowley DE, Jones DN, Eby P, Sanchez CA, Plowright RK. (2018). Changing resource landscapes and spillover of henipaviruses. *Annals of the New York Academy of Sciences*, 1429:78-99. doi: 10.1111/nyas.13910

Korine C, Ishaki I and Arad Z. (1999). Is the Egyptian fruit-bat *Rousettus aegyptiacus* a pest in Israel? An analysis of the bat's diet and implications for its conservation. *Biological Conservation,* 88: 301–306. doi: 10.1016/S0006-3207(98)00126-8

Kranstauber B, Cameron A, Weinzeri R, Fountain T, Tilak S, Wikelski M, Kays R. (2011). The Movebank data model for animal tracking. *Environmental Modelling and Software,* 26: 834–835. doi: 10.1016/j.envsoft.2010.12.005

Krebs CJ. (2009). *Ecology, the Experimental Analysis of Distribution and Abundance*. 6th ed. San Franscisco: Pearson Benjamin Cummings.

Lewin-Koh NJ, Bivand R, Pebesma EJ, Archer E. (2011). *maptools: Tools for Reading and Handling Spatial Objects* R Package Version 0.8-10.

Lowry H, Lill A and Wong BBM. (2013). Behavioural responses of wildlife to urban environments. *Biological Reviews of the Cambridge Philosophical Society,* 88: 537–549. doi:10.1111/brv.12012

Markus N, Hall L. (2004). Foraging behaviour of the black flying fox (*Pteropus alecto*) in the urban landscape of Brisbane, Queensland. *Wildlife Research*, 31(3):345–55. doi: 10.1071/WR01117

McDonald-Madden E, Schreiber ESG, Forsyth DM, Choquenot D and Clancy TF. (2005). Factors affecting Grey-headed flying fox (*Pteropus poliocephalus* : Pteropodidae) foraging in the Melbourne metropolitan area, Australia. *Austral Ecology*, 30: 600-608. doi: 10.1111/j.1442-9993.2005.01492.x

McFarlane RA. (2015). In Health of people, places and planet. Ed CD Butler, J Dixon, AG Capon. Australia National University Press.

Misra P, Burke BP and Pratt M. (1999). GPS Performance in Navigation. In Proceedings of the *Institute of Electrical and Electronics Engineers,* (Special Issue on GPS), 187: 65-85. doi: 10.1109/5.736342

Nakamoto A, Kinjo K and Izawa M. (2007). Food habits of Orii's flying fox, *Pteropus dasymallus*, in relation to food availability in an urban area of Okinawa-jima Island, the Ryukyu Archipelago, Japan. *Acta Chiropterologica,* 9: 237–249. doi: 10.3161/150811007781694372

Nakamoto A, Kinjo K and Izawa M. (2012). Ranging patterns and habitat use of a solitary flying fox (*Pteropus dasymallus*) on Okinawa-jima Island, Japan. *Acta Chiropterologica*, 14: 387–399. doi: 10.3161/150811012X661701

Noonan M et al. (2019). A comprehensive analysis of autocorrelation and bias in home range estimation. *Ecological Monographs*, 89. doi: 10.1002/ecm.1344

Oleksy RZ, Ayady CL, Tatayah V, Jones C, Howey PW, Froidevaux JSP, Racey PA, Jones G. (2019). The movement ecology of the Mauritian flying fox (*Pteropus niger*): a long-term study using solar-powered GSM/GPS tags. *Movement ecology*, 7:12. doi: 10.1186/s40462-019-0156-6

Palmer C, Price O and Bach C. (2000). Foraging ecology of the black flying fox (*Pteropus alecto*) in the seasonal tropics of the Northern Territory, Australia. *Wildlife Research*, 27: 169–178. doi: 10.1071/WR97126

Parris KM and Hazell DL. (2005). Biotic effects of climate change in urban environments: The case of the Grey-headed flying fox (*Pteropus poliocephalus*) in Melbourne, Australia. *Biological Conservation*, 124: 267-276. doi: 10.1016/j.biocon.2005.01.035

Parry-Jones KA and Augee ML. (1991). Food selection by Grey-headed flying foxes (*Pteropus poliocephalus*) occupying a summer colony site near Gosford, New South Wales. *Wildlife Research* 18: 111–124. doi: 10.1071/WR9910111

R Core Team. 2019. R: A language and environment for statistical computing.

Referral guideline for management actions in Grey-headed and Spectacled flying fox camps, Commonwealth of Australia 2015. Available from http://www.environment.gov.au/system/files/resources/6d4f8ebc-f6a0-49e6-a6b6-82e9c8d55768/files/referral-guideline-flying fox-camps.pdf. In effect under the EPBC Act from 06-Dec-2001. Accessed 6 July 2020.

Roberts BJ, Catterall CP, Eby P and Kanowski J. (2012). Long-distance and frequent movements of the flying fox *Pteropus poliocephalus*: implications for management. *PLOS ONE* 7, e42532. doi: 10.1371/journal.pone.0042532

Schmelitschek, E., French, K., and Parry-Jones, K. (2009). Fruit availability and utilisation by Grey - headed flying foxes (Pteropodidae: *Pteropus poliocephalus*) in a human-modified environment on the south coast of New South Wales, Australia. *Wildlife Research*, 36: 592-600. doi: 10.1071/WR08169

Smith CS, Epstein JH, Breed AC, Plowright RK, Olival KJ, de Jong C, Daszak P, Field HE. (2011). Satellite telemetry and long-range bat movements. *PLOS ONE* 6: e14696. doi: 10.1371/journal.pone.0014696

Spencer HJ, Palmer C and Parry-Jones K. (1991). Movements of fruit-bats in eastern Australia, determined by using radio-tracking. *Wildlife Research*, 18: 463-468. doi: 10.1071/WR9910463

Tidemann CR. (1999). Biology and management of the Grey-headed flying fox, Pteropus poliocephalus. *Acta Chiropterologica*, 1: 151–164. doi: 10.7882/fs.2002.04

Tidemann CR and Nelson J (2004). Long-distance movements of the Grey-headed flying fox (Pteropus poliocephalus). *Journal of Zoology*, 263, 141–6. doi: 10.1017/S0952836904004960

Threatened Species Scientific Committee. (2001). *Commonwealth Listing Advice on* Pteropus poliocephalus *(Grey-headed Flying fox)*. Available from: http://www.environment.gov.au/biodiversity/threatened/species/p-poliocephalus.html. In effect under the EPBC Act from 06-Dec-2001. Accessed 6 July 2020.

Threlfall CG, Law B, Banks PB. (2013). Roost selection in suburban bushland by the urban sensitive bat *Nyctophilus gouldi. Journal of Mammalogy* 94: 307–319. doi: 10.1644/11-MAMM-A-393.1

van der Ree R, McDonnell MJ, Temby ID, Nelson J and Whittingham E. (2006). The establishment and dynamics of a recently established urban camp of Pteropus poliocephalus outside their geographic range. *Journal of Zoology*, 268, 177–85. doi: 10.1017/S0952836901000486

Webb NJ and Tidemann CR. (1996). Mobility of Australian flying foxes, Pteropus spp. (Megachiroptera): evidence from genetic variation. *Proceedings of the Royal Society B: Biological Sciences,* 263: 497–502. doi: 10.1098/rspb.1996.0075

Westcott DA, Heersink DK, McKeown A, Caley P. (2015). The status and trends of Australia's EPBC-Listed flying foxes, Technical Report CSIRO, Australia. Accessed December 10th 2020.

Williams NSG, McDonnell MJ, Phelan GK, Keim LD, Van der Ree R. (2006) Range expansion due to urbanization: Increased food resources attract Grey-headed Flying foxes (*Pteropus poliocephalus*) to Melbourne. *Austral Ecology*, 31: 190-198. doi: 10.1111/j.1442-9993.2006.01590.x

Woinarski JCZ, Burbidge AA and Harrison P. (2014). The Action Plan for Australian Mammals 2012, Collingwood, Australia: CSIRO Publishing. Accessed December 10th 2020.

Wood EM and Esaian S. (2020). The importance of street trees to urban avifauna. *Ecological Applications,* 30:7. doi: 10.1002/eap.2149

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Contribution to the Paper	Formulated experimental design. Sample collection and laboratory processing. Co-analysed, co-interpreted, formatted, and edited data. Wrote and revised manuscript		
Overall percentage (%)	70		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature		Date	12.2.21

Chapter 3

DNA Metabarcoding to determine the diet of Grey-headed flying foxes (*Pteropus poliocephalus*) in South Australia.

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3.1 Abstract

Flying foxes in Australia are increasingly using urban and agricultural landscapes as their natural habitat declines in quality and quantity. Characterisation of the diet of Grey-headed flying foxes has previously relied upon observation and the assumed ingestion of nectar and fruit of trees that they have been seen to frequent, along with microscopic examination of faeces. The main objective of this study was to investigate the diet of the newly established Grey-headed flying fox (*Pteropus poliocephalus*) population in Adelaide to understand what foraging resources are allowing the species to persist outside their former distribution range. We collected faeces from captured Grey-headed flying foxes across two years and four sampling periods (August 2016, February 2017, August 2017 and February 2018), extracted plant DNA from the samples, and used two DNA barcodes to identify ingested plants. A total of 40 plant operational taxonomic units (OTUs) were identified from the faeces including 15 orders, 15 families and 10 genera. Over 86% of all plant sequences belonged to just three orders: Rosales (54.35%; including the families Rosaceae and Moraceae, the latter incorporating the genus *Ficus*), Myrtales (21.63%; including the family Myrtaceae which includes the genera *Eucalyptus, Angophora* and *Corymbia*) and Malpighiales (10.26%; including the family Salicaceae which incorporates the genus *Populus*). Exotic species were particularly represented, with over 75% of sequences belonging to OTUs considered alien to South Australia. For example, the percentage of sequences of the genus *Ficus* (figs that are exotic to South Australia but native to other parts of Australia and *Populus* (aspen, cottonwood and poplar trees that are exotic to Australia) was 22.10% and 10.25%, respectively. The family *Myrtaceae* which is native to Australia was represented by 21.56% of all sequences. The relative abundance of sequences for the family *Moraceae* (including figs) was significantly greater in summer than winter. By contrast, the relative abundance of sequences for the family *Myrtaceae* (including Eucalypts), *Arecaceae* (including Palm trees) and *Salicaceae* (including *Populus* species) was significantly greater in winter than summer. Our results suggest that Grey-headed flying foxes have been able to persist in Adelaide as a result of plantings of non-native and exotic vegetation since European settlement.

Keywords: DNA metabarcoding, foraging, barcode, *ndhJ*, *rbcL*, *Pteropus poliocephalus.*

3.2 Introduction

Australian flying foxes (Order: Chiroptera, Family: *Pteropodidae*) provide important ecosystem services as pollinators and seed dispersers across their distribution (Hall and Richards 2000; Fleming et al, 2009). However, the ranges of these species are changing in response to habitat loss (Markus and Hall, 2004; McDonald-Madden et al. 2005; van der Ree et al. 2006), competition for resources (Webb and Tidemann 1995) and other global change phenomena including climate change (Kessler et al. 2018). In some instances, this has resulted in the expansion and establishment of flying fox camps in urban areas (Williams et al. 2006) where food resources can vary from those in their natural habitats.

In 2011, Grey-headed flying foxes (*Pteropus poliocephalus*) expanded their former range and created a camp in Adelaide's Botanic Park, South Australia, a popular recreational site (Schaer et al, 2019). The camp has since increased in size from c. 300 to a maximum of c. 31,000 individuals (L Collins 2021, pers. comm.) through breeding and seasonal immigration, and is now classified as nationally important (Referral guideline, 2015). This increase in camp size has occurred despite emigration and substantial bat mortality during extreme heat waves in the summers of 2017 and 2019. It is possible that the range expansion of Grey-headed flying foxes into South Australia was driven by their need to find alternative food resources (Kessler et al. 2018).

Understanding flying fox foraging (Paez et al, 2018) and diets can provide useful information about the plant species that attract them to new locations including the increased use of urban landscapes. Previously, fruits from 44 species of canopy and edge species mostly from the families Myrtaceae and Moraceae were identified, in north- eastern New South Wales, as food species for Grey-headed flying foxes (Eby 1998). Subsequent research identified Myrtaceae and Proteaceae as dominant

blossom species including 80% identified as eucalypts, and also 46 fruiting species representing 29 families (Eby and Law 2008). In summary, over 187 species from 50 taxonomic families have been recorded as foraging plants for the Grey-headed flying fox across Australia (Williams et al, 2006). An estimated 50% of the species recorded belong to the *Myrtaceae* family, including many species from the order Myrtales which are native to Australia (e.g., *Eucalyptus spp, Corymbia spp, Angophora spp, Callistemon spp and Melaleuca spp*). In contrast, while only 5.3% of the species recorded belong to the *Moraceae* (e.g., figs) which are native to the eastern states of Australia or the exotic *Rosaceae* family (e.g., stone fruits of the genus *Prunus*) within the order Rosales (Williams et al, 2006).

Conventionally, dietary studies for flying foxes in Australasia, Africa and Asia have used telemetry to identify sites where individuals dwell to forage (Eby 1991; Tidemann and Nelson 2004; Breed et al, 2010; Roberts et al, 2012; Choden et al, 2018; Boardman et al, 2020). However, some studies have relied upon direct observation of flying fox feeding behaviour in the field (Parry-Jones and Augee 1991; Eby 1998; Markus and Hall 2004) or the microscopic examination of plant parts in flying fox faeces (Parry-Jones and Augee 1991; Parry-Jones and Augee 2001; Griffith et al, 2020) or the germination of seeds of fruit-remains collected beneath day roosts (Eby 1998). All of these techniques are time consuming and costly, and further microscopic examination may not provide good taxonomic resolution if the specific diagnostic keys are not available to identify the plant species (Aziz et al. 2017). Over recent years there has been a growing interest in using DNA metabarcoding to investigate the diets of animals (Hebert et al, 2003; (Soininen et al, 2013; Kartzinel et al, 2015; Iwanowicz et al, 2016; Camp et al, 2020). DNA metabarcoding can aid in identification of digested plant material in faeces of bats (Hayward, 2013) without requiring the high level of taxonomic expertise necessary for microscopic identification of pollen grains (Pompanon et al, 2012). Plant DNA can be extracted from faeces and target DNA sequences ("barcode") amplified using primers through a polymerase chain reaction (PCR). The resulting DNA amplicons are then

sequenced and matched to taxonomically verified sequences to achieve species identification (Pompanon et al, 2012).

DNA metabarcoding studies that aim to categorise the diets of predatory animals commonly use the universal animal barcode, cytochrome oxidase 1 (CO1) (Jakubaviciute et al, 2017; Leray et al, 2015). In the case of herbivore diets, however, some studies use the plant chloroplast barcodes (*rbcL* and *matK*) to distinguish dietary plants (Craine et al, 2015; Garcia-Robledo et al, 2013). However, *matK* is particularly difficult to amplify in some plant taxa (Heckenhauer et al, 2016; Camp et al, 2020) and it is too long (950bp) to sequence in its entirety using standard next-generation DNA sequencing approaches (Wilkinson et al, 2017). Many researchers have therefore chosen to work with the *rbcL* chloroplast barcode (Garcia-Robledo et al, 2013) and/or shorter non-coding loci such as *trnL* (Craine et al, 2015; Kartzinel et al 2015), ITS2 (Bell et al, 2016; Bell et al, 2019) or *ndhJ* (Bannister et al, 2019; Camp et al, 2020) which specifically delivers high universality and has good discriminating power (CBOL Plant Working Group, 2009; Hollingsworth et al, 2009). To date, metabarcoding studies of plant DNA in flying fox faeces have used the *rbcL* barcode in the Island Flying fox (*Pteropus hypomelanus*) (Aziz et al, 2017) and *ITS2* and *rbcL* barcodes in the Cave Nectar bat (*Eonycteris spelaea*) (Lim et al, 2018).

Here, we used two DNA barcode markers; *rbcL* and *ndhJ*, to identify the plant taxa present in the faeces collected from Grey-headed flying foxes at the Botanic Park camp in Adelaide, South Australia, over four sampling periods across two seasons, summer and winter, between 2016 and 2018. Specifically, we sought to investigate whether Grey-headed flying foxes in Adelaide feed primarily on native plants or exploit introduced plant species as food resources, and to explore any differences in their diet across seasons. We hypothesised that the flying foxes would largely rely on foraging plants that are not native to South Australia.

3.3 Materials and Methods

3.3.1 Ethics

Prior to commencement of this project, we obtained animal ethics approval from The University of Adelaide (S-2015-028) and a wildlife scientific permit from the SA Department of Environment and Water (M-23671-1,2 and 3) to cover the capture and anaesthesia of Grey- headed flying foxes and the collection of faecal samples.

3.3.2 Study site and sample collection

Grey-headed flying foxes arrived in Adelaide in 2011 and since then there has been a constant presence at a camp adjacent to the Adelaide Zoo entrance in Adelaide's Botanic Park. As part of ongoing research into the ecology and health of these bats, bats were captured using high-level mist nets under the camp as they returned from foraging early in the morning. Bats were caught in 2016 and 2017 in February (summer) and August (winter). Captured bats were held in pillowcases/calico bags and taken to Adelaide Zoo to be anaesthetised. Faecal samples were collected from anaesthetised individuals or from the container in which they were held and stored in 5 ml tubes or cryopreservation tubes at -80C until they could be processed. Of the 161 samples collected, 55 were from males and 106 were from females (see Table 1). In addition to the faecal collection, details were recorded of the age, sex, pregnancy and lactation status of each individual. All animals were released within 5 hours of capture at the camp.

Table 1: Summary of faecal samples collected from Grey-headed flying foxes captured from the Adelaide Camp, Botanic Park over four sampling periods between August 2016 and February 2018.

Date	Males	Females	TOTAL
8-10 Aug 2016	18	23	41
8-22 Feb 2017	16	34	50
11-12 Aug 2017	7	18	25
11-13 Feb 2018	14	31	45
			161

3.3.3 DNA extraction

Faecal samples were placed in separate 50mL falcon tubes and were lyophilised in a freeze drier to remove excess moisture (Camp et al, 2020). Samples were then homogenised using a blunt stainless-steel instrument in the falcon tube. 20mg of homogenised material was taken from each sample and placed into separate micro-centrifuge tubes. DNA extraction was performed using the ISOLATE II Plant DNA extraction kit (Bioline, Australia), according to the manufacturer's instructions. DNA was quantified using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc, Australia) and all samples were diluted to 10ng/uL with nanopure water (Sigma, Australia).

3.3.4 DNA metabarcoding of faecal samples

Two DNA barcode markers were selected for this study: *rbcL* due to its relative universality (CBOL Plant Working Group, 2009) and *ndhJ* due its higher taxonomic resolution (Camp et al, 2020). A two-step PCR was used to amplify *rbcL* and *ndhJ* barcodes from faecal samples. First, the following Illumina (Illumina, San Diego, USA) sequences were added to the 5' end of the primers:

F: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG,

R: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG.

Initial amplification was performed in 10uL volumes comprising 1x Biomix (Bioline, Australia), 0.2uM forward and reverse primers, nuclease-free water and 10ng DNA. All reactions were performed in a Biorad T100 Thermal Cycler (Thermo Fisher Scientific Inc, Australia). For the *ndhJ* marker, the protocol was the following: enzyme activation and DNA denaturation at 95°C for 1 minute, followed by 35 cycles of denaturation at 95°C for 15 seconds, an annealing phase at 50°C for 30 seconds and an extension phase at 72°C for 15 seconds. A final extension step was used at 72°C for 5 minutes. The PCR conditions for the *rbcL* were the same except an annealing temperature of 55°C was used. Amplified products were then run on a 1% agarose gel using gel electrophoresis to confirm the presence of DNA amplicons of expected size alongside a 50bp Hyperladder DNA ladder (Bioline, Australia).

PCR products of both barcodes were pooled together for each sample and purified using the Agencourt AMPure XP bead system (Beckman and Coulter, USA), washed with 80% ethanol twice and eluted with 25 µl of nanopure water. Each sample was indexed (coded) with the Nextera XT Index Kit V2 (Set A, B, C and D) (Illumina, San Diego, USA) using two different primers (N7 and S5) to produce a unique and individual index to identify samples after sequencing. Indexing was performed in 12.5 µL volumes using 1x MyFi buffer (Bioline, Australia), 1x MyFi polymerase (Bioline, Australia), 7.0 µl DNA template and 1.25 µl of each indexing primer. The MyFi PCR protocol for indexing involved an enzyme activation phase at 95°C for 1 minute followed by 5 cycles of denaturation 95°C for 15 seconds, annealing at 55°C for 30 seconds and an extension phase at 72°C for 15 seconds. The indexed products for each sample were then bulked together and purified using the Agencourt AMPure XP bead system as detailed above.

To quantify the indexed library the bulked sample was diluted 1: 50 and analysed by real-time PCR (qPCR) using 1x KAPA SYBR FAST qPCR Universal Master Mix (Kapa Biosystems, South Africa), with

0.1 µM forward and reverse primers and 1.0 µl indexed DNA template. A standard curve was produced using three concentrations of PhiX library (Illumina, San Diego, USA) (0.1nM, 0.01nM and 0.001nM) and the qPCR was performed using the Rotor Gene qPCR machine (Corbett, Australia.). Indexed samples were pooled, and 16 pM aliquots were paired-end sequenced on a MiSeq sequencer using a 600-cycle Version 3 kit (Illumina, San Diego, USA). The MiSeq Bcl output files were de-multiplexed and converted to FASTQ files using MiSeq Reporter v2.6 software (Illumina, San Diego, USA).

3.3.5 Bioinformatics

For the *ndhJ* barcode, forward and reverse sequences from each primer/sample set were paired using Mothur software (Schloss et al. 2009) and sequences that could not be joined or produced ambiguous base calls were discarded. For *rbcL*, however, we used the first 220 bp of the forward read only because read lengths did not allow for sufficient sequence for overlap. Primer sequences were then trimmed, and sequences with lengths > 10 bp different to that of the target amplicon length were removed. Identical sequences (PCR duplicates) were collapsed to a single consensus sequence for identification, but sequence counts were retained for relative abundance analysis. Any unique sequences that were present in a single sample only and were observed less than 5 times in that sample were discarded, since such sequences are unlikely to represent key diet species and could potentially result from PCR and/or sequencing error. We blasted sequences against NCBI's non-redundant reference DNA database (https://blast.ncbi.nlm.nih.gov/Blast.cgi), retained the top 20 blast matches, and then used MEGAN v. 6 software to classify sequences to the lowest possible taxonomic unit using the lowest common ancestor (LCA) method (Huson et al, 2016). We defined an operational taxonomic unit (OTU) as a DNA sequence cluster that corresponded to a unique taxonomic group, either order, family, genus or species (Blaxter et al. 2005) and calculated the relative abundance of each OTU for each barcode and sample.

3.3.6 Statistical analysis

To compare seasonal changes in the relative sequence abundance of key families (*Moraceae*, *Myrtaceae*, *Salicaceae* and *Arecaceae* which constituted the largest proportion of sequences identified to family level), we pooled results from both barcodes for each sample. After pooling, we used the sequence count for each family in a sample and the total sequence count for each sample for subsequent analysis. Initially, we fitted a binomial regression model of the proportion of each family's sequences present (with data for each flying fox individual considered a replicate observation) and included survey session as a fixed factor. We then compared the relative abundance of each family between seasons (winter and summer) using planned contrasts. These statistical analyses were run using R software (v. 3.4.0) and functions 'glm' from the 'base' package, and 'glht' from the package 'multcomp'.

3.4 Results

3.4.1 Classification of OTUs and relative abundance of plant taxonomic groups

The mean number of sequences per sample was 40,358 (SE=2,663) and 13,276 (SE=1,094) for the *ndhJ* and *rbcL* primers, respectively. DNA metabarcoding of plant DNA in flying fox faeces classified 40 OTUs belonging to 15 orders, 15 families and 10 genera of plants (*rbcL* = 8, *ndhJ* = 4) (Table 2). At the order level, Rosales accounted for 54.35% of all sequences, while Myrtales and Malpighales accounted for 21.63% and 10.26% of all sequences respectively (Table 2). Together, these orders were represented by 86.24% of all sequences identified from 161 samples over both seasons and all sessions. Over 75% of all sequences could be considered either non-native to South Australia or exotic to Australia, while over 24% of sequences could be considered native to South Australia (Table 2).

Order Rosales. Within this order, figs of the genus *Ficus* (family *Moraceae*), which are not native to South Australia, accounted for 22.10% of all sequences identified across both seasons. However, the *Ficus* genus was only conclusively identified using the *rbcL* marker (74.97% of sequences for that barcode), while a large proportion of unclassified sequences from the Rosales order were detected using the *ndhJ* barcode (58.41 %) which probably represent fig sequences that could not be identified to genus level (Table 2). The *Broussonetia* genus (Paper mulberry, 0.23% of all sequences) was also identified using the *rbcL* primer only. The genus *Prunus* which includes cherries, plums and apricots, all of which are exotic to Australia, was also detected but at low relative abundance using the *rbcL* barcode (0.002% of all sequences).

Order Myrtales. This order was represented by the *Myrtaceae* family but the only genus that could be identified was *Eucalyptus* (0.07% of all sequences), while significant unclassified Myrtaceae sequence was obtained from both barcodes (Table 2).

Order Maliphagales. This order was overwhelmingly represented by the *Salicaceae* family, which is exotic to Australia, and in particular the *Populus* genus, representatives of which include aspen, cottonwood and poplar trees (Table 2).

Other orders: The orders Arecales, Lamiales and Apiales were represented by 4.25%, 3.31% and 0.11% of all sequences respectively (Table 2). Arecales was mostly represented by the family *Arecaceae*, including the genus *Phoenix* (Date palms). Lamiales was mostly represented by the family *Oleaceae,* exotic to Australia, which includes cultivated olives and some privets which were mostly eaten in winter. The Apiales order was dominated by the *Pittosporaceae* family which contains the genera *Pittosporum* and *Billardiera*, both of which are native to Australia. The genera, *Vitis* (Grapes) and Tamarix (*Tamaricaceae* family) were also represented at low levels (Table 2).

3.4.2 Statistical comparisons

Regarding the relative abundance of specific taxa, using the planned seasonal contrast, we found a higher relative abundance of sequences from the *Myrtaceae* family (including Eucalypts) in winter (18.5%) than in summer (3.2%; p <0.001) (Fig 1). Similarly, relative sequence abundances were higher in winter than summer for the *Arecaceae* family (4.2 % c.f. 0.9%; p <0.001) and the *Salicaceae* family (6.5% c.f. 3.0%; p <0.001) (Fig 1). In contrast, relative sequence abundance was lower for the *Moraceae* (includes figs) in winter (4.9%) than in summer (16.9%; p <0.001) (Fig 1). When comparing the relative abundance of specific taxa with sex of the flying fox, there were no statistically significant differences (Fig 2).

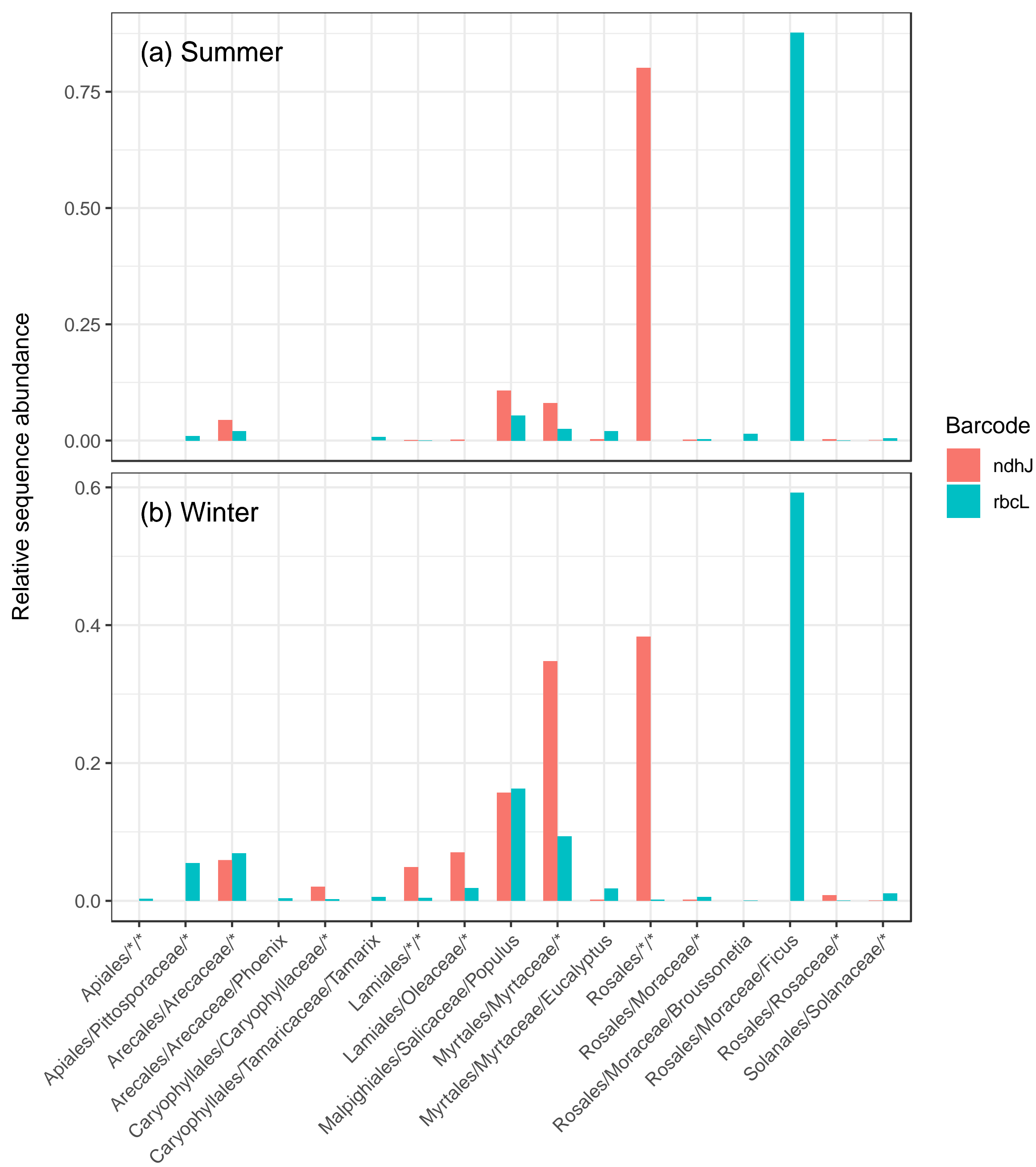

Fig 1: Relative plant sequence abundance at Order/Family/Genus levels in the faeces of Grey-headed flying foxes between August 2016 and February 2018, using primers *rbcL* and *ndhJ* comparing summer and winter. Here, relative abundance was calculated as the average of the sample-level relative abundances. Note the different y-axis scales for each panel. * Indicates unclassified

Table 2: Total number and percentage of total sequences and Relative abundance of plant OTUs from 161 faecal samples collected from Grey-headed flying foxes between August 2016 and February 2018 for the *ndhJ* and *rbcL* barcodes. Where possible, each OTU is classified as native to South Australia, native to Australia but not South Australia, or exotic to Australia. Blank = unknown, ✕ no, ✓= yes

Order	Family	Genus	Common names of representative species (Family and Genus)	Total No Sequences /ndhJ+rbcL	% Of total sequences /ndhJ +rbcL	Relative abundance /mean	Relative abundance /ndhJ	Relative Abundance /rbcL	Native to South Australia	Native to Australia	Exotic to Australia
Rosales[1]				**4,729,559**	**54.35**	**67.62**					
	Moraceae	*Ficus*	Figs	1,923,186	22.11	37.47	0	74.94	✕	✓	✕
	unclassified	unclassified		2,777,961	31.93	29.24	58.41	0.07			
	Moraceae	*Broussonetia*	Paper	19,939	0.23	0.41	0	0.82	✕	✕	✓
	Moraceae	unclassified	Mulberry	8,154	0.09	0.28	0.18	0.38			
	Moraceae	*Maclura*	Osage orange	137	0.001	0.01	0	0.02	✕	✕	✓
	Rosaceae	*Prunus*	Cherries, Plums and Apricots	182	0.002	0.01	0	0.02	✕	✕	✓
Myrtales[1]				**1,882,438**	**21.63**	**10.93**					
	Myrtaceae	unclassified	Myrtles	1,876,250	21.56	10.38	16.92	3.83	✓	✓	✕
	Myrtaceae	*Eucalyptus*	Gums	6,039	0.07	0.54	0.13	0.94	✓	✓	✕
	unclassified	unclassified		149	0.001	0.01	0	0.02			
Malpighiales[1]				**892,535**	**10.26**	**10.69**					
	Salicaceae	*Populus*	Poplar, Aspen and Cottonwoods	892,070	10.25	10.66	12.55	8.78	✕	✕	✓
	Salicaceae	unclassified	Willows	417	0.005	0.02	0	0.03	✕	✕	✓
	unclassified	unclassified		48	0.001	0.01	0	0.02			

Arecales[2]				**369,859**	**4.25**	**3.83**					
	Arecaceae	unclassified	Palm trees	369,497	4.25	3.77	4.44	3.09	✕	⍰	✕
	Arecaceae	*Phoenix*	Date Palm	295	0.003	0.05	0	0.11	✕	✕	⍰
	Arecaceae	*Cocos*	Coconut	267	0.003	0.01	0.01	0	✕	✕	⍰
Lamiales[2]				**287,495**	**3.31**	**1.97**					
	Oleaceae	unclassified	Olives	157,558	1.81	1.2	1.92	0.48	✕	✕	⍰
	unclassified	unclassified		129,937	1.49	0.77	1.4	0.13			
Apiales[1]				**9,783**	**0.11**	**1.11**					
	Pittosporaceae	unclassified	Cheesewoods	9,592	0.11	1.07	0	2.14	⍰	⍰	⍰
	unclassified	unclassified		191	0.002	0.04	0	0.08			
Poales[1]	*Poaceae*	unclassified	Grasses	**230,437**	**2.65**	**0.56**	1.07	0.04	⍰	⍰	⍰
Caryophyllales[1]				**19,187**	**0.22**	**0.54**					
	Caryophyllaceae	unclassified	Pinks	16,831	0.19	0.29	0.53	0.05	⍰	⍰	⍰
	Tamaricaceae	*Tamarix*	Salt cedar	2,329	0.03	0.24	0	0.48	✕	⍰	⍰
	unclassified	unclassified		27	0.0003	0.01	0	0.03			
Solanales[1]	*Solanaceae*	unclassified	Nightshade	**17,016**	**0.20**	**0.28**	0.03	0.53	✕	⍰	⍰
Fabales[1]	*Fabaceae*	unclassified	Legumes	**9,362**	**0.11**	**0.09**	0.02	0.17	✕	⍰	⍰
Asterales[1]	*Asteraceae*	unclassified	Daisies	**15,470**	**0.18**	**0.05**	0.1	0	✕	⍰	⍰
Cucurbitales[1]	*Cucurbitaceae*	unclassified	Gourds	**2,125**	**0.02**	**0.05**	0.04	0.06	✕	⍰	⍰
Vitales[2]	*Vitaceae*	*Vitis*	Grapes	**996**	**0.01**	**0.03**	0.06	0	✕	✕	⍰
Oxalidales[1]	*Oxalidaceae*	unclassified	Wood sorrels	**2,210**	**0.03**	**0.02**	0.04	0	✕	✕	⍰
Unclassified				**222,818**	**2.68**	**2.22**	1.73	2.72			

[1] Angiosperm Phylogeny Group (2009). An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG III. *Botanical Journal of the Linnean Society*. 161 (2): 105–121. doi: 10.1111/j.1095-8339.2009.00996.x

[2] Angiosperm Phylogeny Group (2016). An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG IV. *Botanical Journal of the Linnean Society*, 181: 1–20, doi: 10.1111/boj.12385

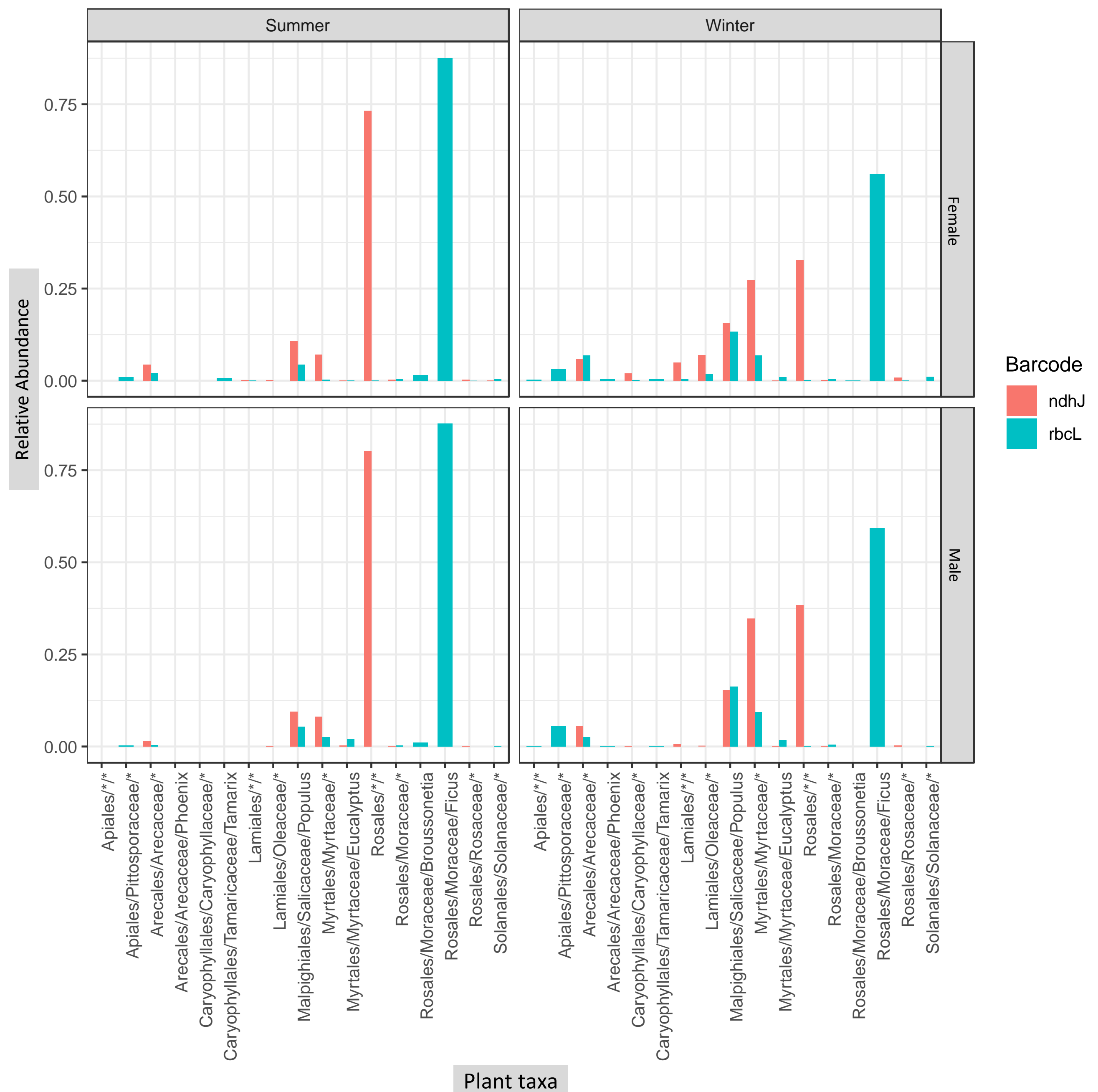

Fig 2: Relative sequence abundance at Order/ Family/Genus levels of plants in the faeces of Grey-headed flying foxes between August 2016 and February 2018, using primers *rbcL* and *ndhJ* comparing summer and winter and sex. Here, relative abundance was calculated as the average of the sample-level relative abundances. * Indicates unclassified

3.5 Discussion

DNA metabarcoding of Grey-headed flying fox faeces collected over two years identified a total of 40 OTUs including 15 orders, 15 families and 10 genera. At the order level, Rosales was represented by 54.35% of all sequences. Over 75% of all sequences were from OTUs either not native to South Australia or exotic to Australia which corroborated our initial hypothesis. The genus, *Ficus*, which is not native to South Australia, from the *Moraceae* family, accounted for at least 22% of all sequences identified. *Ficus* spp such as the Moreton Bay fig (*Ficus macrophylla*) and the Port Jackson fig (*Ficus rubiginosa*) can fruit all year round, while the Common fig (*Ficus carica*) fruits mostly in summer (K Smith 2020, pers.comm.). All of these species, and particularly the Moreton Bay fig, have been planted in Adelaide and surrounding areas since European settlement (K Smith 2020, pers.comm.). We also found some evidence that flying foxes consume species of the *Prunus* genus which includes commercial stone fruits including cherries, plums and apricots. The relative abundance of sequences for families; *Myrtaceae* (e.g., Eucalypts), *Arecaceae* (e.g., palm trees) and *Salicaceae* (e.g., willows and poplars) were significantly greater in winter than in summer, suggesting that flowering and nectar resources belonging to these families are more plentiful and that they contributed a relatively larger part of the diet at that time of the year. By contrast, the relative abundance of sequences for the family *Moraceae* (e.g., figs), was significantly greater in summer than in winter, suggesting fruiting of figs is more abundant and that they contributed a relatively larger part of the diet during this period.

The family *Myrtaceae* was represented by 21.63% of all sequences but only 0.07% of all sequences could be assigned to the genus *Eucalyptus*. The remaining unclassified sequences could be considered native to South Australia although we cannot state that with certainty as many Eucalypts (including genera: *Eucalyptus, Corymbia and Angophora*) have been planted in South Australia since European settlement. A less abundant order, Arecales, was represented by 4.25% of all sequences. Arecales was mostly represented by the family *Arecaceae*, including the genus *Phoenix* (Date palms)

(4.25% of all sequences). The family *Arecaceae* is represented in Australia by some palms including Bangalow palm (*Archontophoenix cunninghamiana*) but many species are exotic including date palms (*Phoenix spp*). The order Lamiales (3.31% of all sequences) was mostly represented by the family *Oleaceae* (1.81% of all sequences), exotic to Australia, which includes cultivated olives and some privets which were mostly eaten in winter by females (Fig.2). Other unclassified Lamiales sequences are likely to be *Myoporum* spp, (*M. floribundum* or *M. montanum*) known to be a foraging plant of Grey-headed flying foxes based on direct observation (L Collins 2021, pers. comm.) in the *Scrophulariaceae* family which is native to Australia. An unexpected finding was that 10.26% of all sequences aligned with the family *Salicaceae* which includes Willows, Aspen, Cottonwood and the genus, *Populus.* While exotic to Australia, the latter genus has been recorded as a foraging tree used by Grey-headed flying foxes (Parry-Jones and Augee 2001). Lombardy poplars (*Populus nigra*) are considered to be a weed but have been planted commonly in South Australia since European times and may have contributed to the *Populus* sequence obtained. Other unexpected findings included 0.01% of all sequences were of the *Vitis* genus (Grape vines), which provides evidence that flying foxes were eating grapes or leaves despite vines growing close to the ground. Other interesting findings included the *Tamarix* genus (*Tamaricaceae* family) (0.03% of all sequences) which is exotic to Australia. Species in this family including *Tamarix parviflora* and *Tamarix ramosissima* are declared pest plants under South Australian Natural Resources Management Act, 2004.

Our research demonstrates that the Grey-headed flying foxes in Adelaide consume plant taxa that are exotic to South Australia in both summer and winter. In theory, DNA metabarcoding can provide greater insights into the diet of flying foxes than the usual microscopic techniques by detecting a wider range of plant taxa, and it can allow identification of plants when no physical plant parts are found in the faeces (Aziz et al, 2017). Unfortunately, however, we were unable to classify many DNA sequences to the genus or species level using the *rbcL* and *ndhJ* barcodes which may be due to an

inadequate reference database (Dormontt et al, 2018) or phylogenetic mismatch due to introgression (Schuster et al, 2018). In contrast to our study, DNA metabarcoding studies on the Island flying fox (*Pteropus hypomelanus*) using the *rbcL* barcode only detected 29 OTUs which were mostly classified to the family level (Aziz et al, 2017), although species-level identification based solely on this barcode proved difficult. Further, a study on the diet of the Cave Nectar bat (*Eonycteris spelaea*) using *ITS2* and *rbcL* primers provided improved taxonomic resolution and was able to detect 55 plant species (Lim et al, 2018).

As in the current study, Aziz et al, (2017) found species-level plant identification based solely on chloroplast DNA metabarcoding is not straightforward (The Consortium for the Barcode of Life, CBOL). Plant Working group recommended barcode loci *rbcL* and *matK* as standard DNA barcode markers, based on the availability of universal primers and the high level of taxonomic resolution (CBOL Plant Working Group, 2009; Hollingsworth et al., 2009). The bar code *ndhJ* can also deliver high universality and has good discriminating power (CBOL Plant Working Group, 2009; Hollingsworth et al., 2009). However, another primer, *trnL* as well as the nuclear ribosomal *ITS2* region have been widely used as DNA barcodes (Sickel et al, 2015), either separately or in combination with *rbcL* (Bell et al, 2017) and *matK* (Kowalczyk et al, 2011). Concerns have been raised that the *rbcL* + *matK* barcodes are less effective because >5% 'false' species-unique barcodes occur (Wilkinson et al, 2017). Additional issues can relate to the effectiveness of the DNA extraction which may vary between food items and some PCR primers may target specific taxa more effectively than others and each PCR from the same extraction is capable of returning different taxa (Alberdi et al, 2019).

The accuracy of DNA barcoding is associated with the taxonomic relatedness of plant species, because related species may have identical DNA sequences in the chosen barcode region (Hollingsworth et al, 2009). Some species cannot be identified at the species level because they may

have identical or similar sequences (Bell et al, 2019) particularly *Eucalyptus* and *Corymbia* species (Schuster et al, 2018), which are common foraging taxa for flying foxes. In fact, only 53% of Australian plant species listed in the Australian Plant Census (12,278/23,057) have been included in reference databases, with only 14% of all species having data for all three of the core DNA barcodes (matK, rbcL, and ITS) (Dormontt et al. 2018). There is also a tendency for barcode loci to fail or only weakly amplify some taxonomic groups (CBOL Plant Working Group, 2011). This produces the opportunity for some dietary components to be omitted or under-represented from inefficiently amplified species (Camp et al, 2020). A further issue for precise plant DNA barcoding in Australia relates to a large number of species in the Myrtaceae family, to regularly hybridise leading to introgression which can lead to phylogenetic mismatch between chloroplast DNA and taxonomic classification (Schuster et al. 2018).

Optimising cut-off scores for taxonomic matching in bioinformatics pipelines can also be a trade-off between maximizing the resolution and minimizing the number of misidentifications (Richardson et al., 2017). A small number of misidentifications can be the result of sequencing error (Bell et al 2019). False results may also occur as a result of cross contamination among samples, index swapping and contamination from the laboratory environment or contaminated reagents (Bell et al, 2019). Furthermore, results can be due to contamination of samples prior to laboratory processing. Typically, these false results cannot be recognised, as negative controls detect only contamination that occurs during the DNA isolation, PCR and sequencing processes (Bell et al, 2019).

Flying foxes have quick gastrointestinal transit times of approximately 30 minutes (Oleksy et al, 2017) or 44 minutes for cultivated fruits (Tedman and Hall, 1985), and while the faeces were collected from anaesthetised animals in our study, the sequences detected by DNA metabarcoding are only likely to reference the forage plants eaten within two-three hours of collection. We know that flying foxes will forage at many sites during the night and so the sequences detected may not

truly reflect what the flying foxes had been eating throughout the entire night only the food items consumed towards the end of the foraging period.

The use of DNA metabarcoding in this study has provided important baseline data for future research into the diet of Australian flying foxes. We stress the importance of developing site-specific or region-specific foraging plant DNA reference database or barcode library to aid in the dietary reconstruction using metabarcoding (Aziz et al, 2017; Camp et al, 2020). We would suggest the use of the barcode *ITS2* in addition to *ndhJ* and *rbcL* for improved taxonomic discrimination (Lim et al, 2018) to the species level. Furthermore, collecting faecal samples weekly throughout the year and over subsequent years will allow for a more detailed construction of the diet and detection of the main taxa ingested by flying foxes in Adelaide over an annual cycle.

In conclusion, we have been able to determine that the Grey-headed flying foxes in Adelaide during summer and winter seasons between 2016 and 2018 were eating foraging species, mostly non-native or exotic to South Australia. This indicates that plantings since European settlement may have provided a 'pull-factor' attracting them into Adelaide in 2011 and subsequently an opportunity for the population to persist and grow.

3.6 Acknowledgements

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3.7 References

Alberdi A, Aizpurua O, Bohmann K, Gopalakrishnan S, Lynggaard C, Nielsen M, Gilbert MTP. (2019) Promises and pitfalls of using high-throughput sequencing for diet analysis. *Molecular Ecology Resources,* 19: 327–348. doi: 10.1111/1755-0998.12960

Aziz SA, Clements GR, Peng LY, Campos-Arceiz A, McConkey KR, Forget P-M, Gan HM. (2017) Elucidating the diet of the island flying fox (*Pteropus hypomelanus*) in Peninsular Malaysia through Illumina Next-Generation Sequencing. *Peer J,* 5, e3176. doi:10.7717/peerj.3176

Bannister H, Croxford A, Brandle R, Paton DC, Moseby K. (2019). Time to adjust: changes in the diet of a reintroduced marsupial after release. *Oryx*, 1-10. doi: 10.1017/S0030605319000991

Bell KL, de Vere N, Keller A, Richardson RT, Gous A, Burgess KS, Brosi BJ. (2016). Pollen DNA barcoding: current applications and future prospects. *Genome*, 59, 629–640. doi: 10.1139/gen-2015-0200

Bell KL, Loeffler VM, Brosi BJ. (2017). An rbcL reference library to aid in the identification of plant species mixtures by DNA metabarcoding. *Applications in Plant Sciences*, 5, 1600110. doi: 10.3732/apps.1600110

Bell KL, Burgess KS, Botsch JC, Dobbs EK, Read TD and Brosi BJ. (2019). Quantitative and qualitative assessment of pollen DNA metabarcoding using constructed species mixtures. *Molecular Ecology*, 28, 431–455. doi:10.1111/mec.14840

Blaxter M, Mann J, Chapman T, Thomas F, Whitton C, Floyd R, Abebe E. (2005). Defining operational taxonomic units using DNA barcode data. *Philosophical Transactions of the Royal Society London B Biological Science,* 360(1462):1935-43. doi: 10.1098/rstb.2005.1725.

Boardman, WSJ, Roshier D, Reardon T, Burbidge K, McKeown A, Westcott DA, Caraguel CGB, and Prowse TAA. 2020. Spring foraging movements of an urban population of Grey-headed flying foxes (*Pteropus poliocephalus)*. *Journal of Urban Ecology*, doi: 10.1093/jue/juaa034

Breed AC, Field HE, Smith CS, Edmonston J, Meers J. (2010). Bats Without Borders: Long Distance Movements and Implications for Disease Risk Management. *EcoHealth*, 7: 204 – 212. doi: 10.1007/s10393-010-0332-z

Camp A, Croxford AE, Ford CS, Baumann U, Clements PR, Hiendleder S, Woolford L, Netzel G, Boardman WSJ, Fletcher MT, Wilkinson MJ. (2020) Dual locus DNA metabarcoding reveals southern hairy nosed wombats (*Lasiorhinus latifrons*) have a summer diet dominated by toxic invasive plants. *PLoS ONE*, 15(3): e0229390. doi: 10.1371/journal.pone.0229390

CBOL Plant Working Group (2009). A DNA barcode for land plants. *Proceedings of the National Academy of Sciences of the USA*, 106, 12794–12797. doi: 10.1073/pnas.0905845106

CBOL Plant Working Group (2011). A DNA barcode for land plants. *Proceedings of the National Academy of Sciences, USA*, 106, 12794–12797. doi: 10.1073/pnas.0905845106

Choden K, Ravon S, Epstein JH, Hoem T, Furey N, Gely M, Jolivot A, Hul V, Neung C, Tran A, Cappelle J. (2019). *Pteropus lylei* primarily forages in residential areas in Kandal, Cambodia. *Ecology and Evolution*, 9: 4181–91. doi: 10.1002/ece3.5046

Craine JM, Towne EG, Miller M, Fierer N. (2015). Climatic warming and the future of bison as grazers. *Nature Science Reports,* 5, Art16738. doi: 10.1038/srep16738

Dormontt EE, van Dijk K, Bell KL, Biffin E, Breed MF, Byrne M, Caddy-Retalic S, Encinas-Viso F, Nevill PG, Shapcott A, Young JM, Waycott M and Lowe AJ. (2018). Advancing DNA Barcoding and Metabarcoding Applications for Plants requires Systematic Analysis of Herbarium Collections—An Australian Perspective. *Frontiers in Ecology and Evolution* 6, 134. doi: 10.3389/fevo.2018.00134

Eby P. (1991). Seasonal movements of Grey-headed flying foxes, *Pteropus poliocephalus* (Chiroptera: Pteropodidae), from two maternity camps in northern New South Wales. *Wildlife Research,* 18: 547–59. doi: 10.1071/WR9910547

Eby P. (1998). An analysis of diet specialization in frugivorous *Pteropus poliocephalus* (Megachiroptera) in Australian subtropical rainforest. *Australian Journal of Ecology*. 23: 443–456. doi: 10.1111/j.1442-9993.1998.tb00752.x

Eby P and Law B. (2008). Ranking the feeding habitat of Grey-headed flying foxes for conservation management. http://www.environment.nsw.gov.au/resources/ threatenedspecies/GHFFmainreport.pdf. Last accessed on November 5, 2019.

Fleming TH, Geiselman C, Kress WJ. (2009). The evolution of bat pollination: a phylogenetic perspective. *Annals of Botany,* 104, 1017–1043. doi:10.1093/aob/mcp197

Garcia-Robledo C, Erickson DL, Staines CL, Erwin TL, Kress WJ. 2013. Tropical Plant-herbivore networks: reconstructing species interactions using DNA barcodes. *PLoS One* 8(1), e52967. doi: 10.1371/journal.pone.0052967

Griffith P, Parry-Jones K and Cunningham AA. (2020). Dietary partitioning in newly sympatric urban flying foxes (*Pteropus poliocephalus* and *Pteropus alecto*). *Australian Mammalogy,* 42, 361-366. doi: 10.1071/AM19047

Hall L and Richards R. (2000). Flying foxes and fruit and blossom bats of Australia. Australian Natural History Series. UNSW Press.

Hayward CE. (2013). DNA barcoding expands dietary identification and reveals dietary similarity in Jamaican frugivorous bats. *Electronic Thesis and Dissertation Repository*. 1697. https://ir.lib.uwo.ca/etd/1697

Heckenhauer J, Barfuss MHJ, Samuel R. (2016). Universal multiplexable matK primers for DNA barcoding of angiosperms. *Applications in Plant Science*, 4(6), 1500137. doi: 10.3732/apps.1500137

Hebert PDN, Cywinska A, Ball SL, deWaard JR. (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society B, Biological Sciences,* 270, 313–321. doi: 10.1098/rspb.2002.2218

Hollingsworth ML, Clark AA, Forrest LL, Richardson J, Pennington RT, Long DG, Cowan R, Chase MW, Gaudeul M, Hollingsworth PM. (2009) Selecting barcoding loci for plants: evaluation of seven candidate loci with species-level sampling in three divergent groups of land plants. *Molecular Ecology Resources,* 9, 439-457. doi: 10.1111/j.1755-0998.2008.02439.x

Huson DH, Beier S, Flade I, Górska A, El-Hadidi M, Mitra S, Ruscheweyh HJ, Tappu R. (2016). MEGAN community edition-interactive exploration and analysis of largescale microbiome sequencing data. *PLOS Computational Biology,* 12(6):e1004957. doi: 10.1371/journal.pcbi.1004957.

Iwanowicz DD, Vandergast AG, Cornman RS, Adams CR, Kohn JR, Fisher RN, Brehme CS. (2016). Metabarcoding of fecal samples to determine herbivore diets: a case study of the endangered pacific pocket mouse. *PLoS ONE*, 11 (11), e0165366. doi: 10.1371/journal.pone.0165366

Jakubaviciute E, Bergstrom U, Eklof JS, Haenel Q, Bourlat SJ. (2017). DNA metabarcoding reveals diverse diet of the three-spined stickleback in a coastal ecosystem. *PLoS ONE,* 12 (10), e0186929. doi: 10.1371/journal.pone.0186929

Kartzinel TR, Chen PA, Coverdale TC, Erickson DL, Kress J, Kuzmina ML, Rubenstein DI, Wang W, Pringle RM. (2015). DNA metabarcoding illuminates dietary niche partitioning by African large herbivores. *Proceedings of the National Academy of Science, USA,* 112 (26), 8019–8024. doi: 10.1073/pnas.1503283112

Kessler MK, Becker DJ, Peel AJ, Justice NV, Lunn T, Crowley DE, Jones DN, Eby P, Sanchez CA, Plowright RK. (2018). Changing resource landscapes and spillover of henipaviruses. *Annals of the New York Academy of Sciences*, 1429:78-99. doi: 10.1111/nyas.13910

Kowalczyk R, Taberlet P, Coissac E, Valentini A, Miquel C, Kamiński T, Wójcik JM. (2011). Influence of management practices on large herbivore diet—Case of European bison in Białowieża Primeval Forest (Poland). *Forest Ecology and Management,* 261: 4, 821-828. doi: 10.1016/j.foreco.2010.11.026.

Leray M, Meyer CP, Mills SC. (2015). Metabarcoding dietary analysis of coral dwelling predatory fish demonstrates the minor contribution of coral mutualists to their highly partitioned, generalist diet. *Peer J*, 3, e1047 doi: 10.7717/peerj.1047

Lim VC, Ramli R, Bhassu S and Wilson J-Jl. (2018). Pollination implications of the diverse diet of tropical nectar-feeding bats roosting in an urban cave. *PeerJ,* 6:e4572. doi: 10.7717/peerj.4572

Markus N, Hall L. (2004). Foraging behaviour of the black flying fox (*Pteropus alecto*) in the urban landscape of Brisbane, Queensland. *Wildlife Research*, 31(3):345–55. doi: 10.1071/WR01117

McDonald-Madden E, Schreiber ESG, Forsyth DM, Choquenot D and Clancy TF. (2005). Factors affecting Grey-headed flying fox (*Pteropus poliocephalus*: Pteropodidae) foraging in the Melbourne metropolitan area, Australia. *Austral Ecology*, 30: 600-608. doi: 10.1111/j.1442-9993.2005.01492.x

Oleksy R, Giuggioli L, McKetterick TJ, Racey PA, Jones G (2017). Flying foxes create extensive seed shadows and enhance germination success of pioneer plant species in deforested Madagascan landscapes. *PLoS ONE*; 12: e0184023. doi: 10.1371/journal.pone.0184023

Paez DJ, Restif O, Eby P and Plowright RK (2018). Optimal foraging in seasonal environments: implications for residency of Australian flying foxes in food-subsidized urban landscapes. *Proceedings of the Royal Society B, Biological Sciences B,* 373: 2017009720170097. doi: 10.1098/rstb.2017.0097

Parry-Jones KA and Augee ML. (1991). Food selection by Grey-headed flying foxes (*Pteropus poliocephalus*) occupying a summer colony site near Gosford, New South Wales. *Wildlife Research* 18: 111–124. doi: 10.1071/WR9910111

Parry-Jones KA and Augee ML. (2001). Factors affecting the occupation of a colony site in Sydney, New South Wales by the Grey-headed Flying fox *Pteropus poliocephalus* (Pteropodidae). *Austral Ecology* 26, 47-55. doi: 10.1111/j.1442-9993.2001.01072.pp.x

Pompanon F, Deagle BE, Symondson WOC, Brown DS, Jarman SN, Taberlet P. (2012). Who is eating what: diet assessment using next generation sequencing. *Molecular Ecology,* 21, 1931-1950. doi: 10.1111/j.1365-294X.2011.05403.x

R Core Team. 2019. R: A language and environment for statistical computing.

Referral guideline for management actions in Grey-headed and Spectacled flying fox camps, Commonwealth of Australia 2015. Available https://www.environment.gov.au/system/files/resources/6d4f8ebc-f6a0-49e6-a6b6-82e9c8d55768/files/referral-guideline-flying-fox-camps.pdf. In effect under the EPBC Act from 06-Dec-2001. (Accessed 10 December 2020).

Richardson RT, Bengtsson-Palme J, and Johnson RM. (2017). Evaluating and optimizing the performance of software commonly used for taxonomic classification of DNA metabarcoding sequence data. *Molecular Ecology Resources,* 17, 760–769. doi: 10.1111/1755-0998.12628

Roberts BJ, Catterall CP, Eby P and Kanowski J. (2012). Long-distance and frequent movements of the flying fox *Pteropus poliocephalus*: implications for management. *PLOS ONE* 7, e42532. doi: 10.1371/journal.pone.0042532

Schaer J, Boardman WSJ, McKeown A, Westcott DA, Mastuschewski K, Power M. (2019). Molecular investigation of Hepatocystis parasites in the Australian flying fox *Pteropus poliocephalus* across its distribution range. *Infection, Genetics and Evolution,* 75, 103978. doi: 10.1016/j.meegid.2019.103978

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF. (2009). Introducing mothur: open-source platform-independent community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology*, 75, 7537–7541. doi: 10.1128/AEM.01541-09

Schuster TM, Setaro SD, Tibbits JFG, Batty EL, Fowler RM, McLay TGB, Wilcox S, Ades PK, Bayly MJ. (2018). Chloroplast variation is incongruent with classification of the Australian bloodwood eucalypts (genus *Corymbia*, family Myrtaceae). *PLoS ONE*, 13(4): e0195034. doi: 10.1371/journal.pone.0195034

Sickel W, Ankenbrand MJ, Grimmer G, Holzschuh A, Hartel S, Lanzen J, Steffan-Dewenter I and Keller A. (2015). Increased efficiency in identifying mixed pollen samples by meta-barcoding with a dual-locus indexing approach. *BMC Ecology,* 15:20. doi: 10.1186/s12898-015-0051-y

Soininen EM, Zinger L, Gielly L, Bellemain E, Brathen KA, Brochmann C, Epp LS, Gussarova G, Hassel K, Henden J-A, Killengreen ST, Rama T, Stenoien HK, Yoccoz NG, Ims RA. (2013). Shedding new light on the diet of Norwegian lemmings: DNA metabarcoding of stomach content. *Polar Biology,* 36, 1069–1076. doi: 10.1007/s00300-013-1328-2

Tedman RA and Hall LS (1985). The morphology of the gastrointestinal tract and food transit time in the fruit bats *Pteropus alecto* and *P. poliocephalus* (Megachiroptera). *Australian Journal of Zoology*, 33,625–640. doi: 10.1071/ZO9850625

Tidemann CR and Nelson J (2004). Long-distance movements of the Grey-headed flying fox (Pteropus poliocephalus). *Journal of Zoology*, 263, 141–6. doi: 10.1017/S0952836904004960

van der Ree R, McDonnell MJ, Temby ID, Nelson J and Whittingham E. (2006). The establishment and dynamics of a recently established urban camp of Pteropus poliocephalus outside their geographic range. *Journal of Zoology*, 268, 177–85. doi: 10.1017/S0952836901000486

Webb NJ and Tidemann CR. (1996). Mobility of Australian flying foxes, Pteropus spp. (Megachiroptera): evidence from genetic variation. *Proceedings of the Royal Society B: Biological Sciences,* 263: 497–502. doi: 10.1098/rspb.1996.0075

Wilkinson MJ, Szabo C, Ford CS, Yarom Y, Croxford AE, Camp A and Gooding P. (2017). Replacing Sanger with next generation sequencing to improve coverage and quality of reference DNA barcodes for plants. *Scientific Reports*, 7, 46040. doi: 10.1038/srep46040

Williams NSG, McDonnell MJ, Phelan GK, Keim LD, Van der Ree R. (2006). Range expansion due to urbanization: Increased food resources attract Grey-headed flying foxes (*Pteropus poliocephalus*) to Melbourne. *Austral Ecology*, 31: 190-198. doi: 10.1111/j.1442-9993.2006.01590.x

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Chapter 4

Seroprevalence of three paramyxoviruses; Hendra virus, Tioman virus, Cedar virus and a rhabdovirus, Australian bat lyssavirus, in a range expanding fruit bat, the Grey-headed flying fox (*Pteropus poliocephalus*).

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4.1 Abstract

Habitat-mediated global change is driving shifts in species' distributions which can alter the spatial risks associated with emerging zoonotic pathogens. Many emerging infectious pathogens are transmitted by highly mobile species, including bats, which can act as spill-over hosts for pathogenic viruses. Over three years, we investigated the seroepidemiology of paramyxoviruses and Australian bat lyssavirus in a range-expanding fruit bat, the Grey-headed flying fox (*Pteropus poliocephalus*), in a new camp in Adelaide, South Australia. Over six, biannual, sampling sessions, we quantified median florescent intensity (MFI) antibody levels for four viruses for a total of 297 individual bats using a multiplex Luminex binding assay. Where appropriate, fluorescence thresholds were determined using finite mixture modelling to classify bats' serological status. Overall, apparent seroprevalence of antibodies directed at Hendra, Cedar and Tioman virus antigens was 43.2%, 26.6% and 95.7%, respectively. We used hurdle models to explore correlates of seropositivity and antibody levels when seropositive. Increased body condition was significantly associated with Hendra seropositivity (Odds ratio = 3.67; p = 0.002) and Hendra virus antibody levels were significantly higher in pregnant females (p = 0.002). While most bats were seropositive for Tioman virus, antibody levels for this virus were significantly higher in adults (p < 0.001). Unexpectedly, all sera were negative for Australian bat lyssavirus. Temporal variation in antibody levels suggests that antibodies to Hendra virus and Tioman virus may wax and wane on a seasonal basis. These findings suggest a common exposure to Hendra virus and other paramyxoviruses in this flying fox camp in South Australia.

4.2 Introduction

The emergence of zoonoses from wildlife represents an increasingly significant threat to global public health (Jones et al, 2008). Bats (Order Chiroptera) are the reservoir host of several significant groups of emerging zoonotic viruses including the paramyxoviruses, (e.g., Hendra virus and Nipah virus), coronaviruses, filoviruses and lyssaviruses (Calisher et al, 2006; Halpin et al, 2011; Smith and Wang, 2013; Wong et al, 2019). In Australia, spill-over of three viruses associated with bats of the genus *Pteropus*, also known as flying foxes, has led to morbidity and mortality in domestic animals and humans. They include two paramyxoviruses, Hendra virus and Menangle virus, and a rhabdovirus, Australian bat lyssavirus [Fraser et al, 1996; Philbey et al, 2008; Field et al, 2011; Barr et al, 2012; Edson et al, 2019). Research into the ecology of these viruses led subsequently to the discovery of several new paramyxoviruses, including Cedar virus, Hervey virus, Yeppoon virus, Grove virus, Teviot virus (Barr et al, 2015) and Tioman virus (Chua et al, 2001). Tioman virus, closely related to Menangle virus (Barr et al, 2015) is the only one of these viruses to be associated with disease. It has been associated with sub-clinical infection in humans and still births and fetal abnormalities in pigs (Yaiw et al, 2007).

The Grey-headed flying fox (*Pteropus poliocephalus*), one of four species of flying foxes found on mainland Australia, is classified nationally as Vulnerable under the *Environment Protection and Biodiversity Conservation Act 1999* (Threatened Species Scientific Committee, 2001). The geographical distribution and migration of Grey-headed flying foxes and other pteropodids is dictated by the distribution and phenology of food plants. These bats regularly move long distances in search of ephemeral floral and fruit resources in native forests (Breed at al, 2010; Kessler et al, 2018). Aggregations of flying foxes can increase rapidly during highly productive flowering events (Eby et al, 1999). Recently, Grey-headed flying foxes were distributed from Ingham in Queensland along the coastal belt of eastern Australia to Melbourne in Victoria. As natural food resources have declined coincident with substantial (*c.* 75%) loss of native forest throughout the south-eastern

coastal areas of Australia, Grey-headed flying foxes have sought alternative food sources, sometimes forming new colonies in urban landscapes (Williams et al, 2006; Tait et al, 2014).

Habitat loss and fragmentation reduce not only the quantity of food available to wildlife, but also the connectivity of foraging patches, particularly if seasonally important resources have been removed (Kessler et al, 2018). In contrast, anthropogenic resource subsidies, which favour monoculture (e.g., fruit orchards) and introduced species, change the composition and seasonality of available food and the overall nutritional landscape (Kessler et al, 2018). Recently, Grey-headed flying foxes formed camps in Canberra and western parts of Victoria and, during 2011, approximately 1300 individuals migrated to Adelaide, South Australia, thereby expanding the former range of the species. Since that time, the population in Adelaide's Botanic Park, which is a popular recreational location, has increased to approximately 20,000 individuals due to births and continued immigration (November 2019), despite seasonal emigration and substantial bat mortality events during extreme heat waves in summer. Concerns have been raised that the bat camp may constitute a biohazard to the public and to domestic animals. Indeed, Australian bat lyssavirus was detected in a Grey-headed flying fox from the camp in 2012 (Cox-Witton, 2019). Since then, another twenty-six Grey-headed flying foxes from the camp were opportunistically tested for the virus of which none tested positive. However, uncertainty remains about the endemicity of Australian bat lyssavirus in the Adelaide camp.

When investigating the infection dynamics of emerging viruses in bat colonies, direct viral detection and identification is important but is technically limited due to restricted distribution of the virus in organs and transient viral shedding in biological fluids. Complementing virus detection, the exposure to specific viruses can be measured by detecting antibodies against those viruses in bat sera. Antibodies are generally present for months or even years even if the virus is scarcely distributed or even after it is cleared from the animal. As a result, viral seroprevalence monitoring has often been

the first line of investigation for emerging bat zoonoses (Breed et al, 2011; Hayman et al, 2008; Plowright et al, 2008; Pourrut et al, 2007; Plowright et al, 2016). However, interpreting serological results is challenging (Epstein et al, 2008) in part due to variation in the magnitude and longevity of antibody responses to different viruses, and the time of collection of serum post infection (Gilbert et al, 2013). Furthermore, antibodies may cross-react with or cross-neutralize related viral antigens, which can limit the specificity of assays.

Serum viral neutralization tests (SNTs) have been considered the reference method for detecting specific antibodies to Hendra virus (Daniels et al, 2001). However, the use of SNTs is logistically constraining because the highest level of biocontainment (Biosafety level 4) is required to maintain the live viral cultures used for the neutralization assays. Instead, IgG enzyme-linked immunosorbant assays (ELISAs) and Luminex based assays (Bossart et al, 2007) have been favoured because they can be performed under standard biosafety conditions (Epstein et al, 2013). Luminex based fluorescent microsphere binding assays (Bossart et al, 2007) are a sensitive method for detection and quantification of antibodies against Hendra and Nipah viruses (Breed et al, 2010; Hayman et al, 2008; Burroughs et al, 2016; McNabb et al, 2014) and Australian bat lyssavirus (Prada et al, 2019) in bat sera. The target antigen for Hendra virus and Cedar virus is recombinant soluble G protein (McNabb et al, 2014) while the target antigens for Tioman virus and Australian bat lyssavirus are nucleoproteins. Luminex assays have been used internationally to detect henipavirus antibodies in bats and other species; including West African fruit bats and domestic pigs [Hayman et al, 2008; Hayman et al, 2011; Peel et al, 2012; Peel et al, 2013), pteropodid bats in Papua New Guinea (Breed et al, 2010) and *Pteropus vampyrus* bats in Indonesia (Sendow et al, 2013).

Serological evidence of infection with Hendra virus has been shown in all four species of pteropodid bat that occur on mainland Australia, throughout their respective ranges (Breed et al, 2011; Edson et al, 2015). There is evidence to suggest that two species, namely the Black flying fox (*Pteropus alecto*)

and the Spectacled flying fox (*P. conspicillatus*), play the most active role in the transmission of Hendra virus to horses (Edson et al, 2015). Hendra virus is shed in the urine, an important vehicle for transmission in Black flying foxes [Edson et al, 2015, Edson et al, 2019; Peel et al, 2019), and the virus has been detected in Grey-headed flying fox uterine fluid which provides evidence for possible transmission at birthing period which lasts from late September to early December (Hall and Richards, 2000) in this species (Williamson et al, 1999; Halpin et al, 2000).

Here, we surveyed the exposure of Adelaide's recently established Grey-headed flying fox population to protein antigens of Hendra virus, Cedar virus, Tioman virus and Australian bat lyssavirus over a three-year period. We used results from Luminex antibody binding assays to develop a finite-mixture model to identify thresholds for defining seropositive flying foxes to characterise seroprevalence for these four viruses. Next, we used a negative-binomial hurdle model and investigated individual-level correlates of (i) seropositivity and (ii) antibody level following seroconversion. We hypothesised that Hendra virus seroprevalence and antibody levels would be associated with reproductive status as previously reported (Breed et al, 2011; Plowright et al, 2008) and that Australian bat lyssavirus seroprevalence would be apparent given the prior finding of an individual carrying the virus in 2012 (Cox-Witton, 2019).

4.3 Materials and methods

We followed the Consortium for the Standardization of Influenza Seroepidemiology (CONSISE) guidelines (Horby et al, 2017) for the reporting of seroepidemiologic studies which presents items that can be used as a checklist of information that should be included in the results of published seroepidemiologic studies, and which can also serve as a guide to items that need to be considered during study design and implementation. Animal Ethics approval was obtained from The University of Adelaide (S-2015-028) and a wildlife scientific permit from the SA Department of Environment and Water (M-23671-1,2 and 3) prior to commencement of this project.

4.3.1 Sampling

4.3.1.1 Study population

The target and source population were the Grey-headed flying foxes from the only known camp in SA (Fig 4.1) and established in Adelaide's Botanic Park [approximate GPS coordinates: 34°54'56 S, 138°36'24 E]. The camp was sampled over six surveys at approximately six-month intervals between August 2015 (winter) and February 2018 (summer), with the aim of trapping > 50 animals per survey, which constituted our study population.

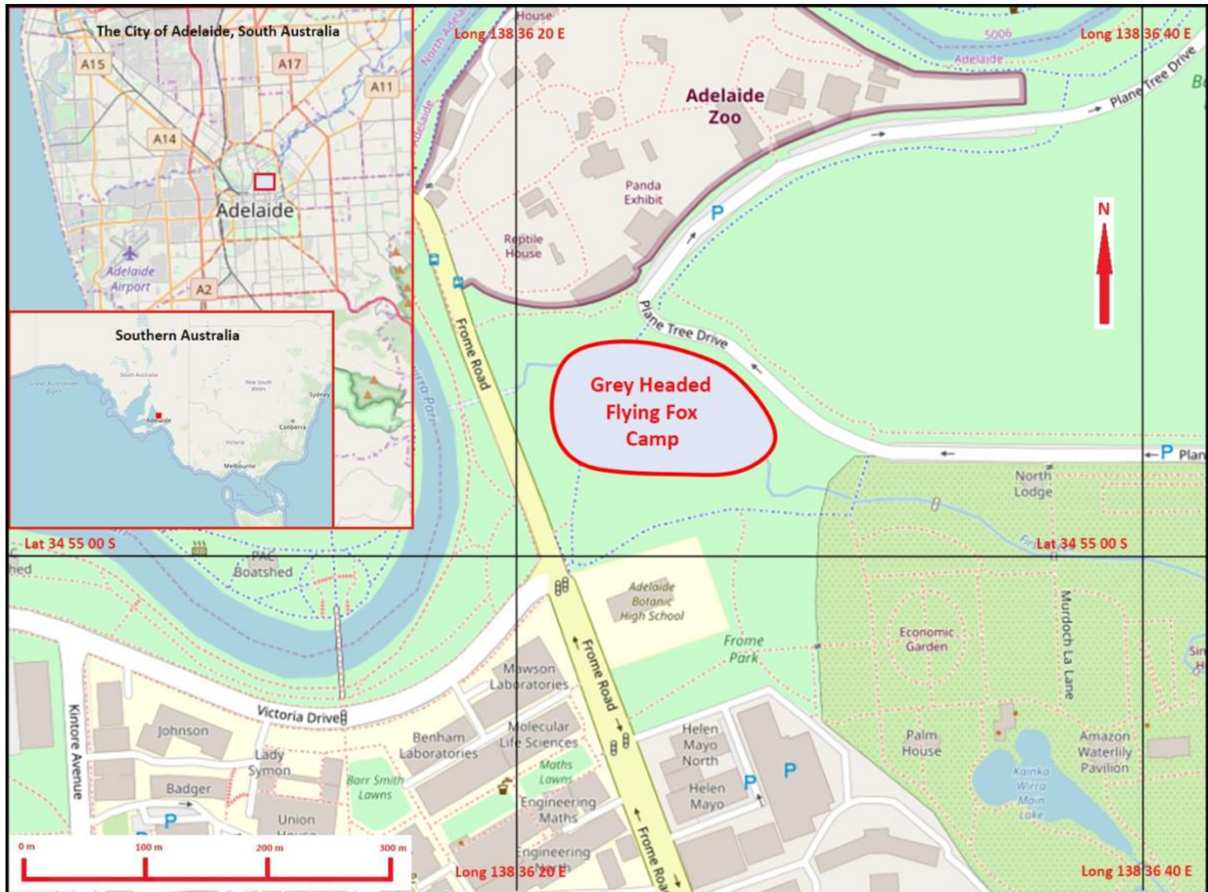

Fig 4.1. Location of the Grey-headed flying fox camp in Adelaide's Botanic Park and relationship to central Adelaide and Southern Australia. Location and extent (red line) of the Grey-headed flying fox camp in Adelaide's Botanic park, showing proximity to Adelaide Zoo where bats were processed. Insets illustrate central Adelaide and Southern Australia to show geographical relationships. Geodata from OpenStreetMap was downloaded via the Maperitive application and the map was rendered with further information supplied by the author.

4.3.1.2 Bat handling, serum and data collection

Study animals were captured at the roost site using 12 or 18 m long mist nets (Ecotone, Gdynia, Poland) installed beneath the camp. Mist nets were raised 20 m above the ground before bats returned from their nightly foraging activity. As each bat became entrapped, the net was lowered, the bat handled with care using thick leather gloves to assure handlers' health and safety, then transferred into pillowcases and relocated to the Animal Health Department of the adjacent

Adelaide Zoo. The net was then elevated to 20 m above the ground to catch further bats. This continued until all bats had returned to the camp. Isoflurane (Isoflurane, Laser Animal Health) was used to anaesthetise bats during data and sample collection following the protocol described by Jonsson et al, 2004. Each bat was permanently identified using a passive integrated transponder tag (Trovan, Microchips Australia Pty, Keysborough, Victoria) inserted subcutaneously between the scapulae. A small amount of fur was clipped from the chest to rapidly identify recaptures at a given survey. In order to prevent dehydration during their short-term confinement, 20-40 mL Hartmann's fluid were injected subcutaneously between the scapulae. Approximately 3-4 mL of blood was collected via venepuncture of the propatagial or brachial vein into 4 mL serum tubes using 22-gauge needles and 3-5 mL syringes. These were allowed to clot overnight at room temperature and then at 4°C before centrifugation (5,000 rpm for 5 minutes) and separation of serum, which was subsequently stored at -80°C. After sampling, bats were placed into pillowcases to fully recover from the anaesthesia before release into the camp.

For each bat, we recorded: (i) sex, (ii) body weight (BW; g), (iii) body condition score (scale of 1 to 5 based on physical palpation of the pectoral musculature by the same person), (iv) forearm length (FAL; mm); elbow to wrist length using vernier callipers, (v) estimated age as described by Hall and Richards [40] (including teeth wear, nipple size for females and enlarged penis/testes for males), (vi) reproductive status (for females; pregnant vs not pregnant by abdominal palpation, lactating vs non lactating by expression of milk and for males; enlarged penis/testes vs small penis/testes). For an objective estimate of the body condition, we also derived a body condition index (BCI) for each individual, calculated subsequently as $BCI = 1,000*(BW/FAL^2)$.

4.3.2 Serology for Hendra virus, Cedar virus, Tioman virus and Rabies virus

Serum samples experienced two freeze/thaw cycles prior to testing. Antibodies against Hendra virus, Cedar virus, Tioman virus, and Australian bat lyssavirus antigens were detected at the Australian

Animal Health Laboratory in Geelong, Victoria using multiplex microsphere assays (Luminex, Austin, USA) as described previously (Bossart et al, 2007). The conformational status of the viruses used were the following; soluble native-like oligomeric G envelope glycoproteins of HeV and CedV (sG_{tet}) were produced from stable expressing FreeStyle™ 293F cell lines (Schulz et al, 2020; Laing et al, 2016), Tioman virus was a nucleocapsid protein expressed in the yeast *Saccharomyces cerevisiae* [Petraityte et al, 2009), and Australian bat lyssavirus was a nucleocapsid protein prepared in *E.coli* (Rahmadane et al, 2017). Briefly, prior to analysis, serum samples were first heat treated at 56°C for 30 minutes to inactivate complement then the assay proteins were coupled to individual microsphere bead sets, of predetermined numbers of magnetic beads, MagPlex® (Luminex, Northbrook, USA). These were added to each well and then mixed with bat sera at a dilution of 1:50. The bound antibody was detected using biotinylated Protein A (Pierce, Rockford, USA) together with biotinylated Protein G (Pierce, Rockford, USA) followed by streptavidin–phycoerythrin (Qiagen Pty Ltd, Australia). The assay was read using a Bio-Plex Protein 200 Array System integrated with Bio-Plex Manager Software (v 6.2) (Bio Rad Laboratories, CA, USA) calibrated on the high setting. Each sample was tested in a well with thousands of beads and the florescence results of 100 beads were recorded as the median florescent intensity (MFI) that excludes outliers and are correlated with antibody concentration. Positivity thresholds for the Luminex serological assay have not been defined for Australian flying foxes due to the lack of negative and single-infection control serum (McNabb et al, 2014) and were therefore estimated using finite mixture modelling (see below).

4.3.3 Statistical analysis

4.3.3.1 Demographic analysis

Two-sample t-test statistics were used to identify any differences in BW, FAL and BCI across demographic classes (sub-adult males and females and adult males and adult pregnant and non-pregnant females) and between winter and summer.

4.3.3.2 Estimating MFI thresholds for classifying seropositive animals

MFI values were log-transformed prior to analysis to approximate a normal distribution and enable parametric analyses. We used finite mixture modelling in the statistical package Stata v15.1 (College Station, Texas, USA) to identify the presence of more than one sub-population under the assumption of normal distribution. Models assuming up to three mixed distributions were run and their parsimony compared using Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC). The model with the lowest AIC and/or BIC was selected as final. When the single distribution model fitted best, the distribution was assumed to be the non-seroconverted bats if MFI values were in the lower end of the range and seroconverted bats if in the higher end of the range. When the two distributions model fitted best, the distributions were considered as the non-seroconverted and the seroconverted bats, respectively, according to their values' range. When the three distributions model fitted best, the distribution with the lowest value range was considered as the non-seroconverted bats and the other two distributions (with higher value ranges) as two sub-groups of seroconverted bats. When two distributions fully overlapped, these were considered as one single distribution because the readings had no discriminative ability. For best fit models with more than one mixed distribution that overlapped partially, threshold values were determined visually at the MFI value for which two predicted normal distributions intersected. These threshold values were used to classify (imperfectly) individual bats as 'seronegative' or 'seropositive' (or 'intermediate positive' when three distributions were identified).

4.3.3.3 Hurdle modelling of seropositivity and antibody levels

To investigate correlates of seropositivity and MFI levels, we used a hurdle regression model which included two components; (i) the 'hurdle' component, which modelled the probability of being seropositive (as defined using the estimated lower threshold value); and (ii) a negative binomial count component, which modelled the antibody value (expressed as MFI) conditional on seropositivity. Explanatory variables investigated were those measured or observed during the

trapping i.e. sex (male or female), age class (subadult < 2.5 years or adult ≥ 2.5 years), body weight (g), forearm length (mm), catching session (1-6), season (winter or summer), pregnancy status, lactation status, and body condition index (BCI). However, we excluded the effect of season from the final model due to strong collinearity between season and time of survey. Hurdle models were implemented within the *R* software for statistical computing (version 3.2.3) using the package *pscl*. The extent of co-seropositivity for all four viruses was also assessed using the negative binomial hurdle regression model investigating the same explanatory variables.

4.3.3.4 Demonstration of zero seroprevalence for Australian bat lyssavirus

The probability that the Adelaide camp is free from Australian bat lyssavirus was estimated using the historical survey analysis outlined by Cameron (2015). The probability of freedom from Australian bat lyssavirus was uncertain before the first survey and a prior value of 50% was used. In the absence of published information on the diagnostic accuracy of the multiplex Luminex assay, we optimistically assumed that this method had perfect accuracy for Australian bat lyssavirus antibodies. Similarly, little information is available on differential risks of Australian bat lyssavirus exposure across bat demographics. Therefore, the risk of Australian bat lyssavirus 'exposure' was assumed constant across the bat camp strata (i.e. risk independent modelling). The 'open' nature of our study population was taken into account by including a 'between-survey' probability of exposure from and/or introduction of immigrating exposed bats into the model. Freedom from Australian bat lyssavirus seroprevalence was deemed achieved if the estimated probability of freedom was ≥ 95%.

4.4 Results

4.4.1 Demographic information

A total of 301 Grey-headed flying foxes were captured over six surveys. Four individual flying foxes were recaptured once each during this period. Demographics including sex, age, weight, forearm

length and BCI varied across seasons reflecting the seasonality of the species' reproduction and feeding opportunities (Table 4.1 and 4.2). Approximately, two thirds were females and two thirds were adults. The overall percentage of adults was similar between sexes (61.7% vs 69.9%). Among the adult females, 31.9% (37/116) were pregnant at capture. Sex and age-class representation was similar across sampling and seasons. Overall the BCI was higher in winter (mean = 28.2, range = 20.2 – 36.1) than summer (mean = 25.3, range = 13.8 – 35.8) (+ 2.9 units, $p < 0.001$) mainly driven by the sub-adult BCI being higher in winter (n = 52, mean = 25.8, range = 20.2 – 29.6) than summer (n=52, mean = 21.1, range = 13.8 – 29.2) (+4.7 units, $p < 0.001$).

Table 4.1. Summary demographic statistics – all captures

Summary demography statistics (number (n), mean, standard deviation (SD), range) of 301 Grey-headed flying foxes captured over six surveys between September 2015 and February 2018 from the Adelaide Camp, Botanic Park, Adelaide. BW = Body weight, FAL = forearm length, BCI = body condition index = 1,000*(BW/FAL^2)

	BW (g)			FAL (mm)			BCI		
Demographic Classes	n	Mean (SD)	Range	n	Mean (SD)	Range	n	Mean (SD)	Range
Sub-adult (all)	106	550 (115.4)	266-772	104	153.0 (7.3)	127.0-171.0	104	23.5 (3.6)	13.8-29.6
Sub-adult Females	72	566 (110.8)	291-739	70	153.8 (6.8)	140.0-170.5	70	23.9 (3.6)	13.8-29.6
Sub-adult Males	34	517 (119.5)	266-772	34	151.7 (7.8)	127.3-162.6	34	22.6 (3.4)	16.4-29.2
Adult (all)	195	764 (90.0)	563-1,008	193	164.0 (4.8)	152.0-176.8	104	23.5 (3.6)	13.8-29.6
Adult Females	116	743 (78.7)	563-1,005	114	163.0 (4.7)	152.0-174.4	114	27.9 (2.8)	22.0-35.1
Pregnant	37	792 (91.6)	600-1005	37	162.3(5.0)	152.0-171.4	37	30.0 (2.7)	22.8-34.9
Not Pregnant	79	720 (60.0)	563-963	79	164.0 (4.6)	154.0-173.0	79	27.0 (2.3)	22.0-35.2
Adult Males	79	794 (96.0)	585-1,008	79	165.2 (4.7)	155.1-176.8	34	29.1 (2.8)	22.6-36.1
All Females	188	676 (126.1)	291-1,005	184	159.5 (7.2)	140.0-173.4	184	26.4(3.7)	13.8-35.2
All Males	113	711 (164.2)	266-1,008	113	160.6 (9.0)	127.3-176.8	113	27.1 (4.2)	16.4-36.1
Total	301	689 (142.4)	266-1,008	297	160.0 (8.0)	127.3-176.8	297	26.7 (3.9)	13.8-36.1

Table 4.2. Summary demographic statistics – winter versus summer

Seasonal demography statistics (number (n), mean, standard deviation (SD), range, winter and summer) of 301 Grey-headed flying foxes captured over six surveys between September 2015 and February 2018 from the Adelaide Camp, Botanic Park, Adelaide. BW = Body weight, FAL = forearm length, BCI = body condition index = 1,000*(BW/FAL)^2

	WINTER									SUMMER								
	BW (g)			FAL (mm)			BCI			BW (g)			FAL (mm)			BCI		
Demographic Classes	n	Mean (SD)	Range	n	Mean (SD)	Range	n	Mean (SD)	Range	n	Mean (SD)	Range	n	Mean (SD)	Range	n	Mean (SD)	Range
Sub-adult	53	629 (42.8)	516-695	52	156.3 (4.1)	146.0-166.0	52	25.8 (2.0)	20.2-29.6	53	472(112.0)	266-772	52	149 (7.8)	127.0-161.0	52	21.1(3.3)	13.8-29.2
Females	40	629 (44.5)	516-695	39	156.2 (4.2)	147.0-166.0	39	25.9 (2.1)	20.2-29.6	32	486 (118.0)	291-739	31	150.5 (8.0)	140.0-170.5	31	21.3 (3.5)	13.8-29.0
Males	13	626 (38.6)	559-675	13	156.4 (3.8)	147.8-162.0	13	25.6 (1.6)	22.7-28.4	21	449 (100.7)	266-772	21	146.1 (7.0)	127.3-162.6	21	20.8 (2.8)	16.4-29.2
Adult	90	790 (99.2)	585-1008	90	163.3 (5.0)	152.0-173.0	90	29.5 (2.9)	22.6-36.1	105	741 (77.3)	563-992	103	164.5 (4.6)	153.5-176.8	103	27.4 (2.4)	22.0-35.8
Females	46	780 (92.5)	600-1005	46	162.1 (4.9)	152.0-171.4	46	29.6 (2.8)	22.8-34.9	70	719 (56.7)	563-963	68	163.8 (4.5)	153.5-173.4	68	26.8 (2.2)	22.0-35.2
Pregnant	37	792 (91.6)	600-1005	37	162.3 (5.0)	152.0-171.4	37	30 (2.7)	22.8-34.9	N/A	N/A	N/A.	N/A.	N/A.	N/A.	N/A.	N/A.	N/A.
Not Pregnant	9	732 (84.9)	645-844	9	161.1 (4.4)	155.1-168.4	9	28.2 (2.8)	24.1-32.6	70	719 (56.7)	563-963	68	164 (4.5)	154.0-173.0	68	26.8 (2.2)	22.0-35.2
Males	44	800 (105.8)	585-1,008	44	164.6 (4.8)	155.1-173.1	44	29.4 (3.0)	22.6-36.1	35	787 (82.9)	622-992	35	165.8 (4.6)	158.1-176.8	35	28.6 (3.1)	24.0-35.8
Total Females	86	710(105.5)	516-1,005	85	159.4 (5.4)	147.0-171.4	85	27.9 (3.1)	20.2-34.9	102	646 (134.7)	291-963	99	160 (8.5)	140.0-173.0	99	25.1 (3.7)	13.7-35.1
Total Males	57	761 (119.9)	559-1,008	57	162.8 (5.7)	147.8-173.1	57	28.6 (3.2)	22.6-36.1	56	660 (187.4)	266-992	56	158.5 (11.1)	127.3-176.8	56	25.7 (4.6)	16.4-35.8
TOTAL	143	730 (113.8)	516-1,008	142	160.7 (5.8)	147.0-173.1	142	28.2 (3.1)	20.2-36.1	158	651 (155.0)	266-992	155	159.2 (9.5)	127.3-176.8	155	25.3 (4.0)	13.8-35.8

4.4.2 Serology thresholds and serological prevalence

Multiplex serology was conducted on 301 serum samples (comprising 297 individual bats, with 4 recaptures). With the exception of Australian bat lyssavirus, these assays yielded multi-modal distributions for log-transformed MFI (Fig 4.2). Three mixed distributions were identified for Hendra virus, Cedar virus and Tioman virus, and two cut-offs, a lower and upper, were determined visually (Fig 4.2). Upper and lower thresholds for the Hendra virus serology were determined as the natural antilogarithms of 5.85 and 8.67, respectively (MFI 347 and 5825, respectively). Upper and lower thresholds for the Cedar virus serology were determined as the natural antilogarithms of 5.76 and 7.44, respectively (MFI 317 and 1702, respectively). Upper and lower thresholds for the Tioman virus serology were determined as the natural antilogarithms of 6.37 and 7.38 respectively (MFI 584 and 1603, respectively). A single distribution of assumed non-seroconverted animals was identified for Australian bat lyssavirus. Using the lower threshold values, 26.6% of the bats were seropositive for Cedar virus, 43.2% of the bats for Hendra virus and 95.7% of the bats for Tioman virus were seropositive (Table 4.3).

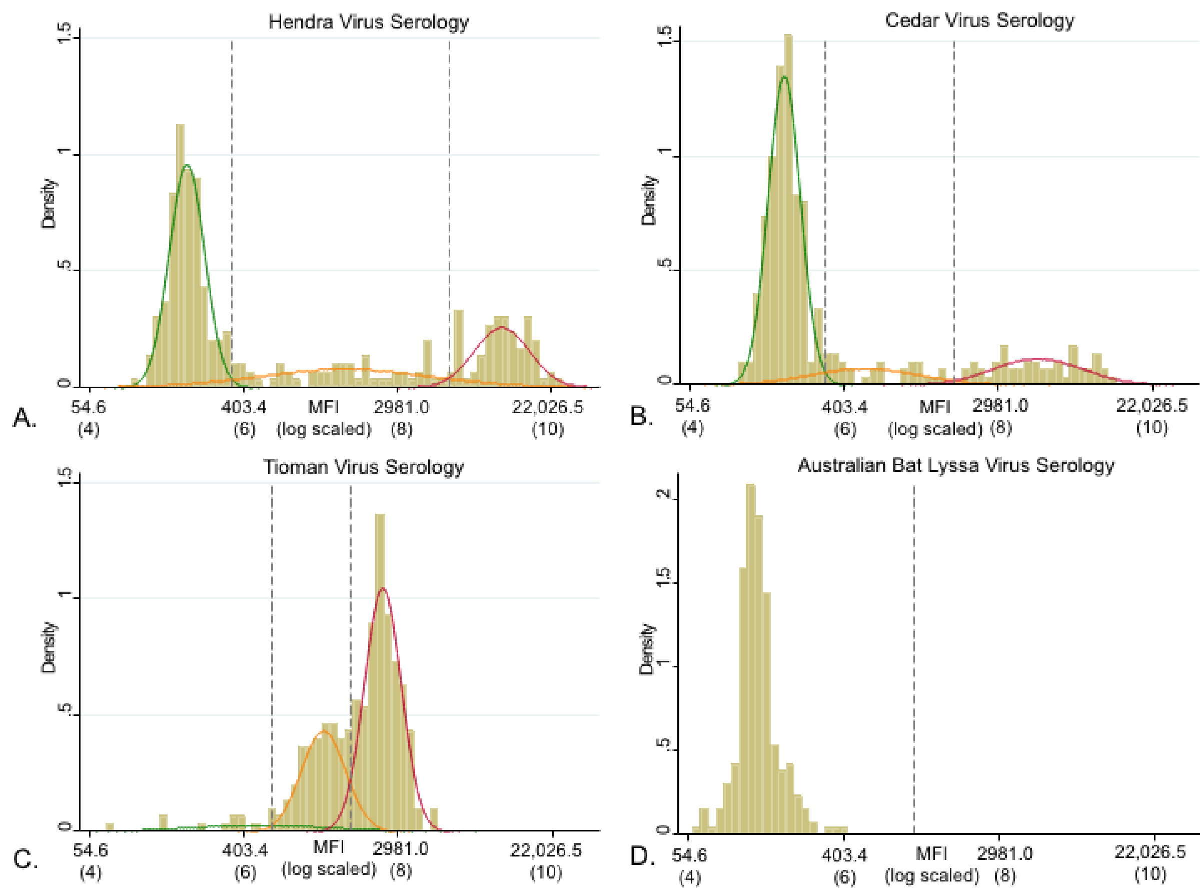

Fig 4.2 Density histogram and overlaid mixture of modelled distributions

Density histogram and overlaid mixture of modelled distributions for MFI and the natural log MFI of A) Hendra virus, B) Cedar virus, C) Tioman virus and D) Australian bat lyssavirus serological readings. Thresholds (dashed vertical lines) correspond to the intersection between a pair of predicted distributions. Upper and lower thresholds for the Hendra virus serology were determined as the natural antilogarithm of 5.85 and 8.67, respectively (MFI 347 and 5,825, respectively). Upper and lower thresholds for the Cedar virus serology was determined as the natural antilogarithm of 5.76 and 7.44 respectively (MFI 317 and 1,702 respectively). Upper and lower thresholds for the Tioman virus serology was determined as the natural antilogarithm of 6.37 and 7.38 respectively (MFI 584 and 1,603 respectively). Upper and lower thresholds could not be calculated for Australian bat lyssavirus as it was determined that all animals belong to the same exposure sub-population.

Table 4.3. Lower threshold MFI scores with corresponding % seroprevalence

Lower thresholds including median fluorescence intensity (MFI) and log MFI and seroprevalence with confidence intervals (CI) for Hendra virus, Cedar virus, Tioman virus and Australian bat lyssavirus for Grey-headed flying foxes sampled in Adelaide, South Australia between September 2015 and February 2018 (n=301). na = not applicable.

Virus	Lower MFI threshold (log MFI)	% seroprevalence (Binomial exact 95% CI)	MFI median values (range) for seropositive animals
Hendra virus	347 (5.85)	43.2% (37.5%-49%)	6,813 (353 – 23,922)
Cedar virus	317 (5.76)	26.6% (21.7%-31.9%)	3,074 (326 – 13,759)
Tioman virus	584 (6.37)	95.7% (92.7%-97.7%)	2,121 (629 – 4,972)
Australian bat lyssavirus	na	0% (0.0%- 1.22%)	na

4.4.3 Hurdle modelling of seropositivity and antibody levels

Using lower threshold levels, the probability of Hendra virus seropositivity was positively and significantly associated with body condition index (Odds ratio = 3.67, p = 0.002). Cedar virus seropositivity was not associated with any of the investigated factors. 95.7% of all bats were Tioman virus seropositive and the hurdle model could not converge because of saturation (Table 4.4 and Fig 4.3). Using the antibody level model, Hendra virus antibody MFI levels were significantly higher in pregnant seropositive females and at the second survey in February 2016 (when 57.4% of individuals were seropositive). However, Hendra virus antibody MFI levels were significantly lower at the fifth survey in September 2017 when 37.0% of individuals were seropositive. Tioman virus MFI antibody levels were significantly higher in adults than sub-adults and at the fifth catching session in September 2017 (Table 4.4 and Fig 4.3).

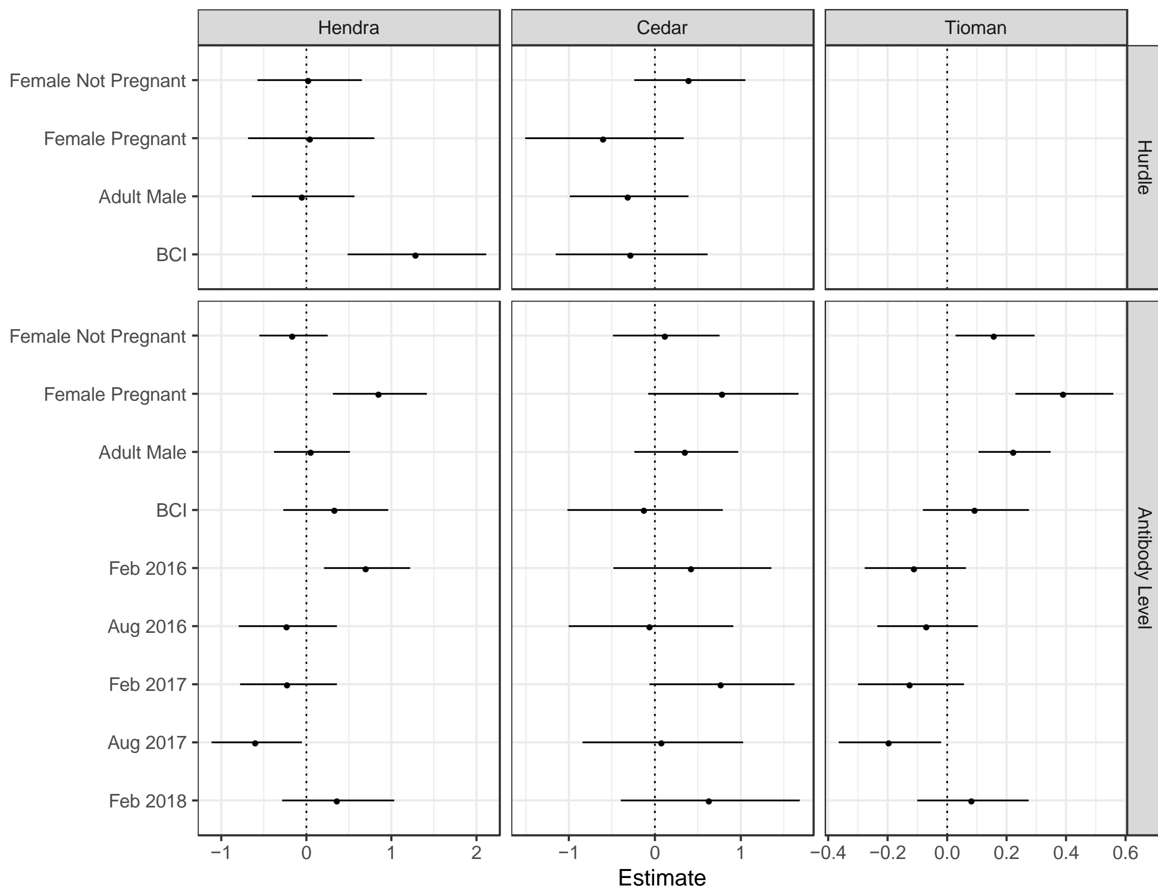

Fig 4.3. Effect plots for hurdle and antibody level models

Effect plots for the estimates and P values for negative binomial hurdle and antibody level models for Hendra virus, Cedar virus and Tioman virus serology for Grey-headed flying foxes sampled between August 2015 and February 2018.

Table 4.4 Statistics for hurdle and antibody level models

Odds ratios (OR), estimates, standard errors (SE), Z values and P values for negative binomial hurdle and antibody level models for Hendra virus, Cedar virus and Tioman virus serology for Grey-headed flying foxes sampled between August 2015 and February 2018. Reference values relate to sub-adults (both male and female) for the hurdle and antibody level model and for the first catching session (August 2015) for the antibody level model. Preg = pregnant. BCI centred = body condition index centred around the mean values. CI = confidence interval.

MODEL	**Hendra virus**					**Cedar virus**					**Tioman virus**				
HURDLE	**Estimate**	**OR (95% CI)**	**SE**	**Z value**	**P value**	**Estimate**	**OR (95% CI)**	**SE**	**Z value**	**P value**	**Estimate**	**OR (95% CI)**	**SE**	**Z value**	**P value**
Intercept	-0.311	-	0.204	-1.526	0.127	-0.996	-	0.221	-4.500	<0.001	.	.	.	.	.
Adult male	-0.036	-1.04	0.308	-0.118	0.906	-0.298	-1.34	0.352	-0.847	0.397	.	.	.	.	.
Female Not Preg	0.040	1.04	0.313	0.127	0.899	0.407	1.50	0.330	1.235	0.217	.	.	.	.	.
Female Preg	0.057	1.05	0.379	0.151	0.880	-0.587	-1.80	0.471	-1.246	0.213	.	.	.	.	.
BCI centred	**1.300**	**3.67**	**0.416**	**3.126**	**0.002**	-0.270	1.31	0.451	-0.598	0.550	.	.	.	.	.

ANTIBODY LEVEL	**Estimate**	**SE**	**Z value**	**P value**	**Estimate**	**SE**	**Z value**	**P value**	**Estimate**	**SE**	**Z value**	**P value**
Intercept	8.717	0.218	40.027	<0.001	7.616	0.347	21.960	<0.001	7.543	0.070	108.318	<0.001
Adult male	0.067	0.228	0.295	0.768	0.365	0.308	1.185	0.236	**0.227**	**0.062**	**3.667**	<0.001
Female Not Preg	-0.150	0.205	-0.732	0.464	0.133	0.316	0.420	0.675	**0.161**	**0.068**	**2.374**	**0.018**
Female Preg	**0.865**	**0.281**	**3.073**	**0.002**	0.797	0.446	1.787	0.074	**0.394**	**0.084**	**4.685**	**0.000**
BCI centred	0.346	0.315	1.099	0.272	-0.113	0.461	-0.245	0.806	0.097	0.091	1.064	0.287
Feb-16	**0.715**	**0.258**	**2.766**	**0.006**	0.436	0.469	0.930	0.353	-0.107	0.087	-1.231	0.218
Aug-16	-0.218	0.295	-0.739	0.460	-0.045	0.489	-0.092	0.927	-0.066	0.086	-0.761	0.447

Feb-17	-0.209	0.291	-0.719	0.472	0.780	0.430	1.813	0.070	-0.122	0.091	-1.341	0.180
Aug-17	**-0.583**	**0.271**	**-2.152**	**0.031**	0.093	0.477	0.194	0.846	**-0.192**	**0.088**	**-2.191**	**0.028**
Feb-18	0.374	0.336	1.113	0.266	0.645	0.531	1.213	0.225	0.087	0.096	0.907	0.365

4.4.4 Investigation of freedom from Australian bat lyssavirus seroprevalence.

None of the tested bats yielded a MFI high enough to imply seroconversion. Accounting for the number of bats captured at each sampling session, there was enough evidence to demonstrate, with 95% confidence, that the Australian bat lyssavirus seroprevalence is less that 2%, assuming that the probability of the camp to be exposed (or an immigrating bat being exposed) between samplings was ≤ 5% (Table 4.5). There was not enough evidence to demonstrate with confidence a seroprevalence ≤ 1% regardless of the probability of exposure.

Table 4.5 Probability of freedom from Australian bat lyssavirus exposure

Summary of final probability of freedom from Australian bat lyssavirus exposure after 6 surveys of a total 301 bats (all seronegative) from the Adelaide Grey-headed flying fox camp. Bold types represent values of probability of freedom where a minimum threshold of 95% was reached.

	Probability of exposure				
Design prevalence (P*)	0.5%	1%	2%	5%	10.0%
1%	94.6%	93.8%	92.2%	87.2%	78.4%
2%	**99.5%**	**99.2%**	**98.6%**	**96.7%**	93.3%
5%	**100.0%**	**99.9%**	**99.8%**	**99.6%**	99.1%
10%	**100.0%**	**100.0%**	**100.0%**	**100.0%**	**99.9%**

4.4.5 Recapture seroprevalence analysis

Over the six sessions, four bats were recaught: three males and one female (Table 4.6). Between survey one and two, September 2015 and February 2016, respectively, the Hendra virus MFI antibody level for one male almost doubled from MFI 9428 to 16929, suggesting exposure occurred prior to September 2015 and continued until February 2016 or reinfection or recrudescence of Hendra virus occurred during this same period. This male's weight also increased as it was classified as a sub-adult in September 2015 and an adult in February 2016. Another male seroconverted for Cedar virus between Sept 2015 and Feb 2016, suggesting exposure occurred during that period. All four animals were seropositive for Tioman virus at both sampling periods while two males did not seroconvert for Hendra virus and thus remained seronegative between the two six-month time

periods. Furthermore, two males and one female did not show evidence of exposure to Cedar virus between sampling periods.

Table 4.6 Seroprevalence changes in recaptured Grey-headed flying foxes

The sex, weight (Wt), body condition index (BCI) and study identification number (Bat ID) of four Grey-headed flying foxes recaptured between August 2015 and August 2017 at the Adelaide Camp, Adelaide, South Australia and their median fluorescence intensity (MFI) serostatus for Hendra virus, Cedar virus, Tioman virus (using lower thresholds) and Australian bat lyssavirus. M= Male; F=Female; Seropositive = +; Seronegative = - .

Bat ID	Date of capture	Sex	Wt /g	BCI	Hendra virus MFI	Hendra virus seropositive	Cedar virus MFI	Cedar virus seropositive	Tioman virus MFI	Tioman virus seropositive	Australian bat lyssavirus MFI	Australian bat lyssavirus seropositive
6 & 72	31 Aug 2015	F	844	29.8	772	-	139	-	2797	+	136	-
	26 Feb 2016		763	26.9	745	-	153	-	2355	+	91	-
23 & 70	2 Sept 2015	M	820	30.1	172	-	181	-	2592	+	130	-
	26 Feb 2016		744	26.6	128	-	3073	+	1248	+	115	-
46 & 81	3 Sept 2015	M	666	25.4	9428	+	175	-	2820	+	92	-
	26 Feb 2016		773	28.0	16929	+	191	-	2979	+	141	-
145 & 252	10 Aug 2016	M	818	30.7	8710	+	257	-	2217	+	204	-
	13 Aug 2017		830	30.8	6335	+	210	-	3111	+	207	-

4.5 Discussion

Our study showed strong evidence of exposure of the Adelaide Grey-headed flying fox camp to Hendra virus, Cedar virus and Tioman virus and no evidence of exposure to Australian bat lyssavirus. The semiquantitative results provided by Luminex binding assays also identified individual-level correlates of seropositivity and antibody levels. Hendra virus seroprevalence in this study (43.2%, 95%CI: 37.5%-49%) is similar to that reported previously (44.5%) (Burroughs et al, 2016) using a Luminex binding assay and compares with an overall seroprevalence of 23.6% using a serum neutralisation test in Little red flying foxes (*P.scapulatus*) (Plowright et al, 2008) and an overall seroprevalence of 56% using a serum neutralisation test in Spectacled flying foxes (Breed et al, 2011). Cedar virus seroprevalence was half than previously reported; 26.6% (95%: 21.7%-31.9%) versus 51.1% in Grey-headed flying foxes (Burroughs et al, 2016). There were some differences in the exposure rates within the camp and across the study sampling times.

Hendra seropositivity was also positively associated with BCI. This contrasts with a previous study (Plowright et al, 2008) which found increased seropositivity in nutritionally stressed Little-red flying foxes but concurs with a study (Edson et al, 2019) in Black flying foxes and Grey-headed flying foxes. In our study, the body condition index of bats was significantly higher in winter than summer (Table 4.2). Food quantity and quality for Grey-headed flying foxes are usually inferior in winter elsewhere in their normal range (Eby et al, 2008). Winter immigration of approximately 5-10,000 extra Grey-headed flying foxes into the Adelaide camp (J Van Weenen 2020, pers. comm.) in 2018 and 2019 suggests that Adelaide is an attractive feeding ground during winter. Other studies indicate acute food shortages may be associated with El Nino/La Nina climate cycles (Giles et al, 2016) leading to nutritionally stressed animals and this may be the driver for the seasonal patterns of Hendra virus seroprevalence (Plowright et al, 2008; Plowright et al, 2015). Late gestation was positively associated with higher Hendra virus MFI antibody levels in comparison to non-pregnant females and males. Similar evidence is seen in serological surveys of Spectacled flying foxes (Breed et al, 2011) and Little

red flying foxes (Plowright et al, 2008) which showed increased detection of Hendra virus antibodies associated with late-stage gestation or early lactation but is in contrast to recent research in Grey-headed flying foxes (Edson et al, 2019) where there is no association.

Hendra virus and Tioman virus seropositivity varied across surveys (Fig 4.3) with Hendra virus seroprevalence significantly increasing between August 2015 and February 2016. This pattern could be explained by: i) “exposure and spread in a sedentary camp” where a Hendra virus exposure event that occurred before August 2015 (seroprevalence = 49%) and resulted in an increase in seroprevalence of captured animals in February 2016 (seroprevalence = 57%) without any further exposure occurring in this period (i.e. within camp spread) and negligible emigration/immigration; or ii) “exposure and re-exposure in a sedentary camp” where additional Hendra virus exposure occurred between the two sampling periods which led to a higher seroprevalence at the second sampling period and negligible emigration/immigration; or iii) “periodic emigration” of non-exposed animals and/or “periodic immigration” of previously exposed flying foxes occurred during this period. Previous studies have suggested that Hendra virus is maintained in flying fox populations through episodic infection in a metapopulation structure, and do not persist endemically within a single population (Plowright et al, 2008). Most hypotheses emphasize horizontal transmission within colonies via urine and other secretions, especially during pregnancy and mating (Plowright et al, 2008), or via migration, with the magnitude of migration affected by the spatial connectivity among colonies, resulting in episodic infection (Wang et al, 2013).

The recaptures of four individuals over the sampling period provided some information on the immunity dynamics of these viruses within this species. Two of these animals were not exposed to Hendra virus and thus remained seronegative between the two captures (six-month time period for both). However, one animal’s Hendra virus seropositive MFI antibody level nearly doubled over a six month time period; between September 2015 and February 2016 which could mean: i) it was

recently exposed just before the first sampling and the antibody level continued to rise in response to the second sampling; ii) it was exposed some time before the first sampling and the antibody level peaked between the two captures and was waning at the second; or iii) it was exposed some time before the first sampling and was re-exposed between captures and mounted a further antibody response. Epstein et al, 2013 suggests maternal antibodies to Hendra virus in Black flying foxes last between 7.5 and 8.5 months and acquired immunity to African henipaviruses may last up to 4 years in adult *Eidolon helvum* adults (Peel et al, 2018) but evidence on Grey-headed flying fox's immune response to viruses is sparse. The antibody level of another seropositive animal waned over one year between August 2016 and 2017, suggesting that the animal was less likely to have become further infected (August 2016 to August 2017). The fourth animal was seropositive, and its antibody level waned over one year (August 2016 to August 2017), suggesting that this animal was unlikely re-exposed during this period.

None of the explored explanatory variables predicted Cedar virus serostatus which is consistent with previous reports (Burroughs et al, 2016). Furthermore, there was no evidence of association between the serostatuses of any pair of viruses. Adult bats showed significantly higher antibody levels against Tioman virus in comparison to subadults which may suggest there is a cumulative age-related antibody response to multiple exposures of the virus. Additionally, immunofluorescent antibody and immunoelectron microscopic data suggested that Tioman virus is antigenically related to Menangle virus (Chua et al, 2001) so it is possible that the high seropositivity to Tioman virus could result from the cross reactivity with Menangle virus exposure.

No evidence of Australian bat lyssavirus exposure was found over our study period despite a previous finding of a positive diagnosis in a Grey-headed flying fox in the Adelaide camp in September 2012 (Cox-Witton, 2019). Previous serological surveys have found a 3.0% Australian bat lyssavirus seroprevalence in flying foxes (95% CI: 1.5-5.8%) (Field, 2018) using the rapid fluorescent

focus inhibition test and 2.9% seroprevalence (95% CI: 1.8–4.5%) in six insectivorous species in Western Australia using a Luminex multiplex binding assay (Prada et al, 2019). Rabies virus neutralising antibodies have been shown to wane in experimentally-infected bats within 6 months after an initial inoculation, but persisted for longer (6–12 months) after a second inoculation of surviving bats (Turmelle et al, 2010). Our results suggest that either (i) Australian bat lyssavirus has not been circulating in the camp over this time period; (ii) seropositivity is very short lived; or (iii) infected flying foxes died suddenly and were thus not sampled at surveys. However, bats are thought to be the ancestral reservoir of lyssaviruses (Badrane et al, 2001) and are the only taxa in which antibodies are detected with sufficient frequency to support serosurveillance (Prada et al, 2019) which could indicate that the virus is unlikely to be circulating in the Adelaide camp.

As with all flying fox camps, the population dynamics can often be very fluid with regular patterns of immigration, emigration and range expansions. Some studies show flying foxes can travel hundreds of kilometres (Breed et al, 2010; Field 2015), moving regularly between different camps over their distribution range. Furthermore, there is evidence that all four species of mainland Australian pteropodids can co-occur in the same camp (Parsons et al, 2010). Range expansions and contractions have been noted in both Black flying foxes and Grey-headed flying foxes (Williams et al, 2006; Tidemann, 1999; van der Ree et al, 2006). The range of Black flying foxes has increased southwards greater than 1000km during the twentieth century (Roberts et al, 2012) and this has been proposed as a possible contributing factor to a contraction of Grey-headed flying foxes' distribution range. In its southernmost distribution, Grey-headed flying foxes now live in the urban environments of Melbourne (van der Ree et al, 2006) and Adelaide. While these areas are not thought to be part of the 'climatic niche' of the species during winter, increased temperature due to the 'urban heat island effect' and climate change may have created an environment that is now tolerable (Parris and Hazell, 2005). Therefore, it is conceivable that through this overlap of flying fox

species, transmission and infection may occur anywhere along the distribution range continuum at any time.

Microsphere assays provide a sensitive method to detect henipavirus antibody binding in fruit bat plasma and serum (Breed et al, 2010; McNabb et al, 2014; Peel et al, 2013). The output of these assays, median fluorescence intensity (MFI), are continuous data and present a challenge in determining meaningful threshold values that categorise bats as seropositive or seronegative (Peel et al, 2013). A MFI > 1,000 for Australian bat lyssavirus has been considered positive and < 250 negative. Our use of mixture models to determine threshold values reflects that of Burroughs et al 2016 (Prada et al, 2019) in that we accept that a single threshold is not possible for the serological profile obtained for the Adelaide bats. We looked for 'natural' groupings of binding activity and used two threshold values to divide these groups into negative, intermediate and positive categories. We recognise that binding in the intermediate category may represent an important intermediate stage in antiviral protection, the shift from a seronegative to a seropositive state or vice versa or may represent a susceptible state. Even using the more specific threshold (MFI 5825, 1702, 1603 for Hendra virus, Cedar virus and Tioman virus, respectively), 25.2% of bats caught from the Adelaide camp showed evidence of prior infection with Hendra virus, 16.6% with Cedar virus and 63.8% with Tioman virus which all suggest common exposures at both the individual and camp level.

We acknowledge certain limitations to our study. The most effective technique to capture bats in the Adelaide camp requires nets to be placed from suitable trees under the camp as they return from foraging. The entire footprint of bat roost trees could not be sampled using a formal random sampling approach because of the topography and may therefore constitute a potential sampling bias. Utilising the same capture sites across the whole study period attempted to standardise any potential sampling bias and protect the comparability of samples.

Conclusion

In contrast to other studies, good body condition rather than nutritional stress was an indicator of increased Hendra virus seroconversion. Substantiating other studies, Hendra virus antibody levels were higher in pregnant females. Unexpectedly, there was no evidence of Australian bat lyssavirus seroconversion. This study highlighted the successful use of a multiplexed Luminex binding assay for serological surveys in flying foxes but also the need to expand the research to include more sampling periods over an annual cycle and to compare with viral presence and diversity.

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4.7 References

Badrane H and Tordo N. (2001). Host Switching in *Lyssavirus* History from the Chiroptera to the Carnivora Orders. *Journal of Virology,* 17: 8096–8104**.** doi: 10.1128/JVI.75.17.8096-8104.2001

Barr JA, Smith C, Marsh GA, Field H, Wang L-F. (2012). Evidence of bat origin for Menangle virus, a zoonotic paramyxovirus first isolated from diseased pigs. *Journal of General Virology,* 93, 2590–2594. doi 10.1099/vir.0.045385-0

Barr J, Smith C, Smith I, de Jong C, Todd S, Melville D, Broos A, Crameri G, Haining J, Marsh G, Crameri G, Field HE, Wang L-F.(2015). Isolation of multiple novel paramyxoviruses from pteropid bat urine. *Journal of General Virology*, 96: 24-29. doi: 10.1099/ vir.0.068106–0

Bossart KN, McEachern JA, Hickey AC, Choudhry V, Dimitrov DS, Eaton BT, Wang L-F. (2007). Neutralization assays for differential henipavirus serology using Bio-Plex Protein array systems. *Journal of Virological Methods,* 142: 29–40. doi: 10.1016/j.jviromet.2007.01.003.

Breed AC, Yu M, Barr JA, Crameri G, Thalmann CM, Wang LF. (2010). Prevalence of henipavirus and rubulavirus antibodies in pteropid bats, Papua New Guinea. *Emerging Infectious Disease*, 16: 1997–1999. doi: 10.3201/eid1612.100879.

Breed AC, Breed MF, Meers J, Field HE. (2011). Evidence of Endemic Hendra Virus Infection in flying foxes (*Pteropus conspicillatus*)—Implications for Disease Risk Management. *PLOS ONE*,6 (12). doi: 10.1371/journal.pone.0028816

Burroughs AL, Durr PA, Boyd V, Graham K, White JR, Todd S, Barr J, Smith I, Baverstock G, Meers J, Crameri G, Wang L-F. (2016). Hendra virus infection dynamics in the Grey-headed flying fox (*Pteropus poliocephalus*) at the southern-most extent of Its range: further evidence this species does not readily transmit the virus to horses. *PLOS ONE*, 11 (6) doi: 10.1371/journal.pone.0155252

Calisher CH, Childs JE, Field HE, Holmes KV and Schountz T. (2006). Bats: important reservoir hosts of emerging viruses. *Clinical Microbiology Reviews,* 19, 531–545. doi: 10.1128/CMR.00017-06

Cameron A, Njeumi F, Chibeu D. and Martin T. (2014). Risk-based disease surveillance. Food and Agriculture Organization of the United Nations. Available from. https://agris.fao.org/agris-search/search.do?recordID=XF2015004263. Sourced December 1 2019.

Chua KB, Wang LF, Lam SK, Crameri G, Yu M, Wise T.(2001). Tioman virus, a novel paramyxovirus isolated from fruit bats in Malaysia. *Virology,* 283: 215– 229. doi: 10.1006/viro.2000.0882

Cox-Witton K. Australian bat lyssavirus Bat Stats. Wildlife Health Australia website. Available from https://www.wildlifehealthaustralia.com.au/Portals/0/Documents/ProgramProjects/ABLV_Bat_Stats_Jun_2019.pdf. Accessed November 2019

Daniels P, Ksiazek T, Eaton BT. (2001). Laboratory diagnosis of Nipah and Hendra virus infections. *Microbes and Infection,* 3: 289–295. doi: 10.1016/S1286-4579(01)01382-X

Eby P, Richards G, Collins L and Parry-Jones K. (1999). The distribution, abundance and vulnerability to population reduction of a nomadic nectarivore, the Grey-headed flying fox, *Pteropus poliocephalus*, in New South Wales, during a period of resource concentration. *Australian Zoologist*, 31, 240–53. doi: 10.7882/AZ.1999.024

Eby P and Law B. (2008). Ranking the feeding habitat of Grey-headed flying foxes for conservation management. http://www.environment.nsw.gov.au/resources/threatenedspecies/GHFFmainreport.pdf. 2008 Last accessed on November 5, 2019.

Edson D, Field H, McMichael L, Vidgen M, Goldspink L, Broos A, Melville D, Kristoffersen J, de Jong C, McLaughlin A, Davis R, Kung N, Jordan D, Kirkland D, Smith C. (2015). Routes of Hendra virus excretion in naturally infected flying foxes: implications for viral transmission and spillover risk. *PLOS ONE*. 10 (10): e0140670. doi: 10.1371/journal.pone.0140670

Edson D, Peel AJ, Huth L, Mayer DG, Vidgen ME, McMichael L, Broos A, et al, Melville D, Kristoffersen J, de Jong C, McLaughlin A, Field HE. (2019). Time of year, age class and body condition predict Hendra virus infection in Australian black flying foxes (*Pteropus alecto*). *Epidemiology and Infection*, Volume 147, e240. doi: 10.1017/S0950268819001237.

Epstein JH, Prakash V, Smith CS, Daszak P, McLaughlin AB, Meehan G, Field HE, Cunningham AA. (2008). Henipavirus infection in fruit bats (*Pteropus giganteus*), India. *Emerging Infectious Disease,* 14: 1309–1311. doi: 10.3201/eid1408.071492

Epstein JH, Baker ML, Zambrana-Torrelio C, Middleton D, Barr JA, Dubovi E, Boyd V, Pope B, Todd S, Crameri G, Walsh A, Fielder MD, Davies AJ, Wang L-F, Daszak P. (2013). Duration of maternal antibodies against canine distemper virus and Hendra virus in Pteropid bats. *PLOS ONE,* 8(6): e67584. doi: 10.1371/journal.pone.0067584

Field HE, de Jong C, Melville D, Smith C, Smith I, Broos A, Kung YH, McLaughlin A, Zeddeman A. (2011). Hendra virus infection dynamics in Australian fruit bats. *PLOS ONE* 6: e28678. doi: 10.1371/journal.pone.0028678

Field HE. (2018). Evidence of Australian bat lyssavirus in diverse Australian bat taxa. *Zoonoses and Public Health,* 65: 742-748. doi: 10.1111/zph.12480

Fraser GC, Hooper PT, Lunt RA, Gould AR, Gleeson LJ, Hyatt AD, Russell GM, Kattenbelt JA. (1996). Encephalitis caused by a lyssavirus in fruit bats in Australia. Emerging Infectious Disease, 2; 327-31. doi: 10.3201/eid0204.960408

Gilbert A, Fooks AR, Hayman DTS, Horton DL, Muller T, Plowright RA, Peel AJ, Bowen R, Wood JLN, Mills J, Cunningham ZZ, Rupprecht CE. (2013). Deciphering serology to understand the ecology of infectious diseases in wildlife. *Ecohealth,* 10: 298-313. doi: 10.1007/s10393-013-0856-0

Giles JR, Plowright RK, Eby P, Peel AJ, McCallum H. (2016). Models of Eucalypt phenology predict bat population flux. *Ecology and Evolution,* 6: 7230–7245. doi: 10.1002/ece3.2382

Hall LS and Richards G. (2000). Flying foxes: Fruit and Blossom Bats of Australia. University of New South Wales Press, Sydney

Halpin K, Young PL, Field HE, McKenzie JS. Isolation of Hendra virus from pteropid bats: a natural reservoir of Hendra virus. *Journal of General Virology*, 1: 1927–1932. doi: 10.1099/0022-1317-81-8-1927

Halpin K, Hyatt AD, Fogarty R, Middleton D, Bingham J, Epstein JH, Rahman SA, Hughes T, Smith CS, Field HE, Daszak P and the Henipavirus Ecology Researrch Group. (2011). Pteropid bats are confirmed as the reservoir hosts of henipaviruses: a comprehensive experimental study of virus transmission. *American Journal of Tropical Medicine and Hygiene,* 85: 946–951. doi: 10.4269/ajtmh.2011.10-0567

Hayman DTS, Suu-Ire R, Breed AC, McEachern JA, Wang L, Wood JLN, Cunningham AA. (2008). Evidence of henipavirus infection in west African fruit bats. *PLOS ONE,* 3 (7): e 2739. doi: 10.1371/journal.pone.0002739

Hayman DTS, Wang LF, Barr J, Baker KS, Suu-Ire R, Broder CC, Cunningham AA, Wood JLN. (2011). Antibodies to henipavirus or henipa-like viruses in domestic pigs in ghana, West Africa. *PLOS ONE*, 6 doi: 10.1371/journal.pone.0025256

Horby PW, Laurie KL, Cowling BJ, Engelhardt OG, Sturm-Ramirez K, Sanchez JL, Katz JM, Uyeki TM, Wood J, Kerkhove MD, CONSISE Steering Committee. (2017). CONSISE statement on the reporting of Seroepidemiologic Studies for influenza (ROSES-I statement): an extension of the STROBE statement. *Influenza and Other Respiratory Viruses,* 11: 2-14. doi: 10.1111/irv.12411

Jones KE, Patel NG, Levy MA, Storeygard A, Balk D, Gittleman JL, Daszak P. (2008). Global trends in emerging infectious diseases. *Nature,* 451: 990–993. doi: 10.1038/nature06536

Jonsson NN, Johnston SD, Field H, de Jong, C, Smith C. (2004). Field anaesthesia of three Australian species of flying fox. *Veterinary Record,* 154: 664. doi: 10.1136/vr.154.21.664

Kessler MK, Becker DJ, Peel AJ, Justice NV, Lunn T, Crowley DE, Jones DN, Eby P, Sanchez CA, Plowright RK. (2018). Changing resource landscapes and spillover of henipaviruses. *Annals of the New York Academy of Sciences*, 1429:78-99. doi: 10.1111/nyas.13910

Laing E, Yan L, Sterling S, Broder C. (2016). A Luminex-based multiplex assay for the simultaneous detection of glycoprotein specific antibodies to ebolaviruses, marburgviruses, and henipaviruses. *International Journal of Infectious Disease,* 53: 108-109. doi: 10.1016/j.ijid.2016.11.272

McNabb L, Barr J, Crameri G, Jusva S, Colling A, Riddell S, Colling A, Boyd V, Broder CC, Wang L-F, Lunt R. (2014). Henipavirus microsphere immune-assays for detection of antibodies against Hendra virus. *Journal of Virological Methods*, 200: 22-28. doi: 10.1016/j.jviromet.2014.01.010

Parris K and Hazell D. (2005). Biotic effects of climate change in urban environments: The case of the Grey-headed flying fox (Pteropus poliocephalus) in Melbourne, Australia. *Biological Conservation* 124: 267–276. doi: 10.1016/j.biocon.2005.01.035

Parsons JG, Van Der Wal J, Robson SKA, Shilton LA. (2010). The implications of sympatry in the Spectacled and Grey headed flying fox, *Pteropus conspicillatus* and *P. poliocephalus* (Chiroptera: Pteropodidae). *Acta Chirop terologica*, 12: 301–309. doi: 10.3161/150811010X537882

Peel AJ, McKinley TJ, Baker KS, Barr JA, Crameri G, Hayman DTS, Feng Y-R, Broder CC, Wang L-F, Cunningham AA, Wood JLN. (2013). Use of cross-reactive serological assays for detecting novel pathogens in wildlife: Assessing an appropriate cutoff for henipavirus assays in African bats. *Journal of Virological Methods*, 193: 295–303. doi: 10.1016/j.jviromet.2013.06.030

Peel AJ, Baker KS, Hayman DTS, Broder CC, Cuningham AA, Fooks AR, Garnier R, Wood JLN, Restif O. (2018). Support for viral persistence from age-specific serology and models of immunity. *Nature Scientific Reports*. doi: 10.1038/s41598-018-22236-6

Peel AJ, Wells K, Giles J, Boyd V, Burroughs A, Edson D, Crameri G, Baker ML, Field HE, Wang L-F, McCullum H, Plowright RK, Clark N. (2019). Synchronous shedding of multiple paramyxoviruses coincides with peak periods of Hendra virus spillover. *Emerging Microbes and Infections,* 8: 1314-1323, doi: 10.1080/22221751.2019.1661217

Petraityte R, Tamosiunas PL, Juozapaitis M, Zvirbliene A, Sasnauskas K, Shiell B, Russell G, Bingham J, Michalski WP. (2009). Generation of Tioman virus nucleocapsid-like particles in yeast *Saccharomyces cerevisiae*. *Virus Research*, 145:92–96. doi: 10.1016/j.virusres.2009.06.013

Philbey AW, Kirkland PD, Ross AD, Davis RJ, Gleeson AB, Love RJ, Daniels PW, Gould AR, and Hyatt AD. (1998). An apparently new virus (family Paramyxoviridae) infectious for pigs, humans, and fruit bats. *Emerging Infectious Disease,* 4:269-271. doi: 10.3201/eid0402.980214

Plowright RK, Field HE, Smith C, Divljan A, Palmer C, Tabor G, Dazsak P, Foley JE. (2008). Reproduction and nutritional stress are risk factors for Hendra virus infection in little red flying foxes (*Pteropus scapulatus*). *Proceedings of the Royal Society B: Biological Sciences*, 275: 861 – 869. doi: 10.1098/rspb.2007.1260

Plowright RK, Eby P, Hudson PJ, Smith IL, Westcott DA, Bryden WL, Middleton D, Reid PA, McFarlane RA, Martin G, Tabor GM, Skerratt LF, Anderson DL, Crameri G, Quammen D, Jordan D, Freeman P, Wang L-F, Epstein JH, Marsh GA, Kung NY, McCallum H. (2015). Ecological dynamics of emerging bat virus spillover. *Proceedings of the Royal Society B: Biological Sciences*, 282: 20142124. doi: 10.1098/rspb.2014.2124

Plowright RK, Peel AJ, Streicker DG, Gilbert AT, McCallum H, Wood J, Baker ML, Restif O. (2016). Transmission or Within-Host Dynamics Driving Pulses of Zoonotic Viruses in Reservoir-Host Populations. *PLOS Neglected Tropical Diseases*, 10 (8). doi: 10.1371/journal.pntd.0004796

Pourrut X, Delicat A, Rollin PE, Ksiazek TG, Gonzalez JP, Leroy EM. (2007). Spatial and temporal patterns of Zaire ebolavirus antibody prevalence in the possible reservoir bat species. *Journal of Infectious Disease*, 196 S176-S183. doi: 10.1086/520541

Prada D, Boyd V, Baker M, Jackson B, O'Dea M. (2019). Insights into Australian Bat Lyssavirus in Insectivorous Bats of Western Australia. *Tropical Medicine and Infectious Disease*, 4: 46. doi: 10.3390/tropicalmed4010046

Rahmadane I, Certoma AF, Peck GR, Fitria Y, Payne J, Colling A, Shiell BJ, Beddome G, Wilson S, Yu M, Morrissy C, Michalski WP, Bingham J, Gardner IA, Allen JD. (2017). Development and validation of an immunoperoxidase antigen detection test for improved diagnosis of rabies in Indonesia. *PLOS Neglected Tropical Diseases*, 11(11): e0006079. doi: 10.1371/journal.pntd.0006079

Roberts BJ, Catterall CP, Eby P, Kanowski J. (2012). Latitudinal range shifts in Australian flying foxes: a re-evaluation. *Austral Ecology,* 37: 12–22. doi: 10.1111/j.1442-9993.2011.02243.x

Schulz JE, Seifert SN, Thompson JT, Avanzato V, Sterling SL, Yan L, Letko MC, Matson MJ, Fischer RJ, Tremeau-Bravard A, Seetahal JFR, Ramkissoon V, Foster J, Goldstein T, Anthony SJ, Epstein JH, Laing Ed, Broder CC, Carrington CVF, Schountz T, Munster VJ. (2020). Serological Evidence for Henipa-like and Filo-like Viruses in Trinidad Bats. *Journal of Infectious Disease,* 4: S375–S382. doi: 10.1093/infdis/jiz648

Sendow I, Ratnawati A, Taylor T, Adjid RM, Saepulloh M, Barr J, Wong F, Daniles P, Field HE. (2013). Nipah virus in the fruit bat Pteropus vampyrus in Sumatra, Indonesia. *PLOS ONE*, 8 (7): e69544. doi: 10.1371/journal.pone.0069544

Smith I and Wang L-F. (2013). Bats and their virome. An importance source of emerging viruses capable of infecting humans. *Current Opinion in Virology*, 3: 84–91. doi: 10.1016/j.coviro.2012.11.006

Tait J, Perotto-Baldivieso HL, McKeown A, Westcott DA. (2014). Are flying foxes coming to town? Urbanisation of the spectacled flying fox (Pteropus conspicillatus) in Australia. PLOS One, 9(10). doi: 10.1371/journal.pone.0109810

Threatened Species Scientific Committee (2001). *Commonwealth Listing Advice on* Pteropus poliocephalus *(Grey-headed flying fox)*. Available from: http://www.environment.gov.au/biodiversity/threatened/species/p-poliocephalus.html. In effect under the EPBC Act from 06-Dec-2001. Accessed 6 November 2019.

Tidemann CR and Nelson J (2004). Long-distance movements of the Grey-headed flying fox (Pteropus poliocephalus). *Journal of Zoology*, 263, 141–6. doi: 10.1017/S0952836904004960

Tidemann CR. (1999). Biology and management of the Grey-headed flying fox, Pteropus poliocephalus. *Acta Chiropterologica*, 1: 151–164. doi: 10.7882/fs.2002.04

Turmelle AS, Jackson FR, Green D, McCracken GF, Rupprecht CE. (2010). Host immunity to repeated rabies virus infection in big brown bats. *Journal of General Virology*, 91: 2360–2366. doi: 10.1099/vir.0.020073-0

van der Ree R, McDonnell MJ, Temby ID, Nelson J and Whittingham E. (2006). The establishment and dynamics of a recently established urban camp of Pteropus poliocephalus outside their geographic range. *Journal of Zoology*, 268, 177–85. doi: 10.1017/S0952836901000486

Wang HH, Kung NY, Grant WE, Scanlan JC, Field HE (2013). Recrudescent infection supports Hendra virus persistence in Australian flying fox populations. *PLOS ONE*, 8: doi: 10.1371/journal.pone.0080430

Williams NSG, McDonnell MJ, Phelan GK, Keim LD, Van der Ree R. (2006) Range expansion due to urbanization: Increased food resources attract Grey-headed flying foxes (*Pteropus poliocephalus*) to Melbourne. *Austral Ecology*, 31: 190-198. doi: 10.1111/j.1442-9993.2006.01590.x

Williamson MM, Hooper PT, Selleck PW, Westbury HA, Slocombe RF. (1999). Experimental Hendra virus infection in pregnant guinea-pigs and fruit bats (*Pteropus poliocephalus*). *Journal of Comparative Pathology,* 122201–7. doi: 10.1053/jcpa.1999.0364

Wong ACP, Li X, Lau SKP, Woo PCY. (2019). Global Epidemiology of Bat Coronaviruses. *Viruses.* 2019; 11: 174. doi: 10.3390/v11020174

Yaiw KC, Bingham J, Crameri G, Mungall B, Hyatt A, Yu M, Eaton B, Shamala D, Wang L-F, Wong KT. (2007). Tioman Virus, a paramyxovirus of bat origin, causes mild disease in pigs and has a predilection for lymphoid tissues. *Journal of Virology,* 2007; 82: 565-568. doi: 10.1128/JVI.01660-07

Statement of Authorship

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Chapter 5

Evidence of exposure to Coronaviruses and to a Filovirus in a range-expanding species: the Grey-headed flying fox (*Pteropus poliocephalus*) in South Australia.

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Keywords: range expansion, seroepidemiology, Ebola Zaire virus, SARS-CoV-1, MERS-CoV, Luminex binding assay

5.1 Abstract

Coronaviruses and filoviruses are emerging pathogens that have caused several disease outbreaks including the ongoing pandemic caused by Severe acute respiratory syndrome virus 2 (which causes the human disease termed Covid – 19). Bats have been implicated as reservoir hosts for many coronaviruses and filoviruses but evidence flying foxes play a role in the transmission dynamics of these viruses is currently lacking. In this study, we followed-up on the serosurvey reported in Chapter 4 and investigated the seroepidemiology of protein antigens to Ebola Zaire virus, Severe acute respiratory syndrome virus (SARS-CoV-1) and Middle Eastern respiratory syndrome virus (MERS-CoV) in the Grey-headed flying fox (*Pteropus poliocephalus*) camp in Adelaide, South Australia. The same 301 serum samples collected over six, biannual sampling sessions, were screened using a multiplex Luminex binding assay using nucleocapsid proteins to all three viruses and median fluorescence intensity thresholds for defining seropositivity were determined using finite mixture modelling. We estimated the true seroprevalence of antibodies directed at nucleoprotein antigens to Ebola Zaire virus and SARS-CoV-1 as 26.7% (CI: 19.9%-34.4%) and 42.5% (CI: 34.3%-51.2%), respectively. However, we found insufficient evidence of prior exposure to MERS-CoV-like antigen in the flying fox camp. Exposure of the flying foxes in Adelaide to Ebola Zaire virus and SARS-CoV-1 could have occurred anywhere along the multi-species flying fox distribution range continuum.

Key words: seroprevalence, Ebola Zaire virus, SARS-CoV-1, MERS-CoV, Luminex binding assay

5.2 Introduction

Long identified as likely reservoirs of zoonotic pathogens, bats have been associated with numerous emerging infectious viruses, including Henipaviruses, Ebola viruses, Severe acute respiratory syndrome coronavirus (SARS-CoV-1) and Middle Eastern respiratory syndrome coronavirus (MERS-CoV) (Plowright et al, 2016; Holz et al, 2018) and in 2019-2020, Covid – 19 (SARS-CoV-2) (Andersen et al, 2020). Global change processes such as habitat destruction and climate change are driving shifts in the distribution of species and also alter the spatial risks of co-associated viruses (Carlson et al, 2020). Bats, considered viral reservoirs, are predicted to account for the majority of novel virus sharing that could facilitate future emergence in humans particularly in areas of high human population density (Carlson et al, 2020). In Australia, fruit bats from the genus *Pteropus* have been identified as the reservoir host of Hendra virus and Menangle virus (Field et al 2011; Philbey et al, 2008; Barr et al, 2012; Boardman et al, 2020) and considerable research has been undertaken to investigate the viral disease ecology and transmission dynamics (Breed et al, 2011; Field et al, 2011; Edson et al, 2015, Edson et al, 2019). However, our understanding of the occurrence and prevalence of other viral taxa, including coronaviruses and filoviruses, in Australian bats is limited (Smith et al, 2016; Prada et al, 2019).

Coronaviruses are critical emerging pathogens of humans and domestic animals and have caused outbreaks of Severe acute respiratory syndrome (SARS) and Middle East Respiratory Syndrome (MERS), avian infectious bronchitis, transmissible gastroenteritis and porcine epidemic diarrhea (de Groot et al, 2013; Banerjee et al, 2019), and most recently a new severe acute respiratory syndrome (known as COVID-19) associated with SARS-CoV-2 virus (Andersen et al, 2020). Coronaviruses are classified into four genera, alphacoronavirus (alphaCoV), betacoronavirus (betaCoV), gammacoronavirus and deltacoronavirus. Only alphaCoVs and betaCoVs are found in bats (Cui et al, 2007; Anthony et al, 2017), whereas gammacoronaviruses and deltacoronaviruses are primarily found in birds (Banerjee et al, 2019). Within betaCoV, they are further classified into subgenera;

embecovirus, hibecovirus, merbecovirus (including MERS CoV), nobecovirus and sarbecovirus (including SARS-CoV-1 and SARS CoV-2) (ICTV 2019). Many of the human and animal coronaviruses appear to have an origin in bats (Trivedi et al, 2019; Latinne et al, 2020) which corroborates the virus-host coevolution hypothesis (Cui et al, 2007; Anthony et al, 2017) which asserts that many coronaviruses co-evolved with bats. These viruses have a high frequency of recombination (Drexler et al, 2014) and high mutation rates, and like other RNA viruses they may adapt rapidly to new ecological and host niches (Holmes et al, 2004) allowing host shifting to occur (Smith et al, 2016; Leopardi et al, 2018, Wong et al, 2019) which can lead to disease epidemics. To date, over 200 novel coronaviruses have been identified in bats (Chen et al, 2010, Banerjee et al, 2019) and genetically diverse alphaCoVs and betaCoVs have been detected in bats in many continents including Asia (Lau et al, 2010; Li et al, 2016; Lau et al, 2018) and Australasia (Smith et al, 2016; Jeong et al, 2017).

Filoviruses are also often considered zoonotic pathogens, some of which have caused episodic outbreaks of haemorrhagic disease among humans and non-human primates (Sanchez et al, 2002; Bempong et al, 2019). The family *Filoviridae* contains RNA viruses and comprise four genera, including two genera of zoonotic importance: *Marburgvirus*, which includes *Marburg* virus and *Ravn* virus, and *Ebolavirus*, which contains six distinct species including *Bundibugyo, Reston, Sudan, Tai Forest, Bombali* and *Zaire* viruses (Wang and Anderson, 2019). Pathogenicity varies among *Ebola viruses*, from *Zaire* virus, which is highly lethal in humans, to *Reston* virus which causes disease in pigs and macaques but asymptomatically infects humans (Olival et al, 2013). A third genus, *Cuevavirus*, contains only one species, *Lloviu* virus, which was detected in the Common bent-wing bat (*Miniopterus schreibersii*) in Spain (Negredo et al, 2011). A fourth genus, *Dianlovirus*, contains *Mengla* virus and has recently been characterised from *Rousettus* bats in China (Yang et al, 2017).

Anti-ebolavirus antibodies have been detected in three African fruit bat species (the Hammer-headed bat *Hypsignathus monstrosus,* Franquet's epauletted fruit bat *Epomops franqueti,* Little

collared fruit bat *Myonycteris torquate*; Leroy et al, 2005; Pourrut et al, 2009), in Leschenault's rousette *(Rousettus leschenaultia)*, *Cynopterus* spp and Greater false vampire bat (*Megaderma lyra)* in Bangladesh (Olival et al, 2013) and in Chinese bat species (Yuan et al, 2012; He et al, 2015; Yang et al, 2017; Zhang et al, 2020). These studies provide evidence that Ebola virus exposure occurs in bats from mainland Africa, Asia and Europe and confirms the observation that the reservoirs for filoviruses exist across a much larger geographic range than Africa, where the majority of recent human Ebola haemorrhagic disease has occurred (Negredo et al, 2011; Olival and Hayman, 2014).

Because of the sensitive nature of the Ebola Zaire serology results, publication is under embargo at the time of thesis submission. Instead, we published the results on paramyxoviruses and ABLV serology (Chapter 4) first and then published on SARS-CoV-1 and MERS-CoV separately (because of the significance of the findings; see List of included publications arising from this research, page 13 of this thesis). For the purpose of the thesis, we used the samples collected in Chapter 4 to investigate if the Grey-headed flying foxes in Adelaide had been exposed to nucleocapsid antigens of Ebola Zaire virus and, the coronaviruses, SARS-CoV-1 and MERS-CoV. Using the same methodologies reported in Chapter 4, we used the results from Luminex antibody binding assays to develop a finite-mixture model to identify seroprevalence thresholds and then we used a negative-binomial hurdle model to investigate individual-level correlates of a) seropositivity and b) antibody level following seroconversion. *A priori*, we hypothesised that filovirus and coronavirus seroprevalence would be nil in this flying fox population.

5.3 Materials and methods

The same 301 sera collected from the Grey-headed flying foxes in the Adelaide camp were used and analysed using the same methodology as reported in Chapter 4. The same analytical approach was also used to estimate the thresholds for median fluorescence intensity (MFI) and to classify animals and construct a hurdle model of seropositive animals and antibody levels conditional on

seropositivity. We present below a brief overview of the serology and analytical methodologies covered in detail in Chapter 4 as well as original analytical approaches used for this work.

5.3.1 Serology for Ebola Zaire virus, SARS-CoV-1 and MERS-CoV

Collected sera were screened at the Australian Centre for Disease Preparedness in Geelong, Victoria using recombinant Ebola Zaire N, SARS-CoV-1 N (Biorbyt orb 171606) and MERS-CoV N nucleocapsid proteins (produced in *E. coli* and purified directly from SDS-PAGE gels) (Yu et al., 2008) in an indirect binding multiplex sphere assay (Luminex, Austin, USA) (Bossart et al., 2007). In the absence of confirmed thresholds for Ebola Zaire virus, SARS-CoV-1 and MERS-CoV in bat serum, we used methods developed previously (Burroughs et al, 2016; Peel et al, 2013) to establish a MFI threshold value using finite mixture modelling.

5.3.2 Estimation of true prevalence for prevalent viruses

For viruses where the apparent seroprevalence was not null (i.e. prevalent), we corrected for test misclassification by calculating the true seroprevalence using the apparent seroprevalence, diagnostic sensitivity (DSe) and diagnostic specificity (DSp) using the following formula (Dohoo et al, 2009)

$$True\ Seroprevalence = \frac{Apparent\ Seroprevalence + DSp - 1}{DSe + DSp - 1} \quad \textit{(Eq. 1)}$$

We implemented the calculation using the Epitools online platform to obtain 95% confidence interval as well (Epitools, 2020a). For each virus, the diagnostic sensitivity (DSe) was estimated as the fraction of the distribution of the seroconverted flying foxes which was classified as seropositive (i.e. MFI value ≥ the defined threshold) and the diagnostic specificity (DSp) as the fraction of the

distribution of non-seroconverted flying foxes which was classified as seronegative (i.e. MFI value < the defined threshold).

5.3.3 Determination of freedom of exposure for non-prevalent viruses

For viruses where there was no evidence of seroconversion (i.e. non-prevalent), we estimated the probability of the flying foxes to be free from viral exposure based on the achieved sample size and the accuracy of the assay estimated with DSe and DSp as described above. The estimation of the probability of freedom from a given virus was implemented, we used the *Freecalc* analysis function from the Epitools online platform to perform this estimation (Epitools, 2020b).

5.4 Results

5.4.1 Serological thresholds and seroprevalence

The bimodal distribution of the 301 log-MFI values for both Ebola Zaire virus and SARS-CoV-1 suggested a mixture of two normal distributions from two sub-populations of seroconverted and non-seroconverted flying foxes. We selected an MFI threshold at the intersection of the two modelled distributions for each of the viruses (Fig 5.1). this results in estimated MFI thresholds of 544.0 and 497.7 for Ebola Zaire virus and SARS-CoV-1 seropositivity, respectively. The selected threshold for Ebola Zaire virus corresponded to an estimated DSe of 65.1% (95% CI: 47.5%-75.2%)) and DSp of 97.9% (95% CI: (94.3%-99.5%). For SARS-CoV-1 serology, the selected threshold corresponded to a DSe of 67.3% (95% CI:53.2%-99.2%) and a DSp of 94.8% (95% CI: 86.1%-99.2%). No evidence of a mixed distribution was found for MERS-CoV.

A total of 57 bat sera were classified as seropositive to Ebola Zaire virus, providing an apparent seroprevalence of 18.9% (Clopper-Pearson exact 95% CI: 14.7% - 23.8%). After accounting for the imperfect test accuracy, we estimated the true seroprevalence for the Ebola Zaire virus antigen in

the camp to be 26.7% (Clopper-Pearson exact 95% CI: 19.9% - 34.4%) (Table 5.3). A total of 95 bat sera had an MFI which were classified as seropositive to SARS-CoV-1 virus providing evidence that 31.6% of bats had antibodies reactive to SARS-CoV-1 antigen (Clopper-Pearson exact 95% CI: 26.4% - 37.1%). After accounting for the imperfect test accuracy, we estimated the true seroprevalence against the SARS-CoV-1 antigen to be 42.5% (Clopper-Pearson exact 95% CI: 34.3% - 51.2%) (Table 5.2) in the camp.

In contrast, there was no evidence of bi-modality in log-MFI values for reactivity against the MERS-CoV antigen (Fig 5.1). Therefore, we used the laboratory's analytical threshold for seropositivity of MFI ≥ 1,000 (Crameri et al, 2015) which was set such that 97.5% of the non-seroconverted standards would be classified as seronegative. Accordingly, we assumed a DSp of 97.5% for this threshold and allocated a DSe sensitivity of 100.0%. Two bats had MFI > 1,000 which translated into an apparent seroprevalence for MERS-CoV of 0.7% (Clopper-Pearson exact 95% CI: 0.01%-2.4%). True seroprevalence calculation was not possible because the number of seropositive bats was too low. The *FreeCalc* calculation (Epitools) indicated that there was insufficient evidence (confidence < 0.01%) of prior exposure to MERS-CoV-like antigen in the camp (Table 5.1).

Table 5.1: Median fluorescence intensity (MFI) thresholds, screening results, seroprevalence estimation and analysis of camp freedom from exposure, for Ebola Zaire virus, SARS-CoV-1 and MERS CoV for Grey-headed flying foxes in Adelaide, South Australia, Australia, 2015-2018

Features	Ebola Zaire serology	SARS-CoV-1 serology	MERS-CoV serology
MFI threshold			
Selection method	Finite mixture modelling	Finite mixture modelling	Analytical (provided by laboratory)
MFI threshold value (natural antilog)	544 (6.30)	498 (6.21)	1,000 (6.91)
Diagnostic sensitivity	65.1% (47.5%, 75.2%)	67.3% (53.2%, 76.0%)	100.0%[#]
Diagnostic specificity	97.9% (94.3%, 99.5%)	94.8% (86.1%, 99.2%)	97.5%[#]
Screening results			
Number of screened bat sera	301	301	301
Number of seropositive sera	57	95	2
Range of seropositive MFI levels	565 - 4627	498 - 4447	1018 - 1444
Seroprevalence estimation			
Apparent seroprevalence (95% CI)	18.9% (14.7%, 23.8%)	31.6% (26.4%, 37.1%)	0.7% (0.01%, 2.4%)
True seroprevalence (95% CI)[*]	26.7% (19.9%, 34.4%)	42.5% (34.3%, 51.2%)	na
Analysis of camp freedom from exposure using FreeCalc[**]			
Estimation method	Modified hypergeometric exact	Modified hypergeometric exact	Modified hypergeometric exact
Design prevalence (the hypothetical seroprevalence to be detected)	5%	5%	5%
Type-I-error (false negative camp exposure)	5%	5%	5%
Type-II-error (false positive camp exposure)	5%	5%	5%
Exposure confidence (probability of observing this many positive bats or more if the camp was truly exposed)	>99.99%	>99.99%	<0.01%
Number of positive bats required to consider the camp exposed given the sample size and assay accuracy	≥ 12	≥ 23	≥ 13

[*] Estimating prevalence calculator available at https://epitools.ausvet.com.au/trueprevalence.

[**]FreeCalc calculator available at https://epitools.ausvet.com.au/freecalcone. na – not achievable

[#]theorical values for which 95% CIs are not relevant

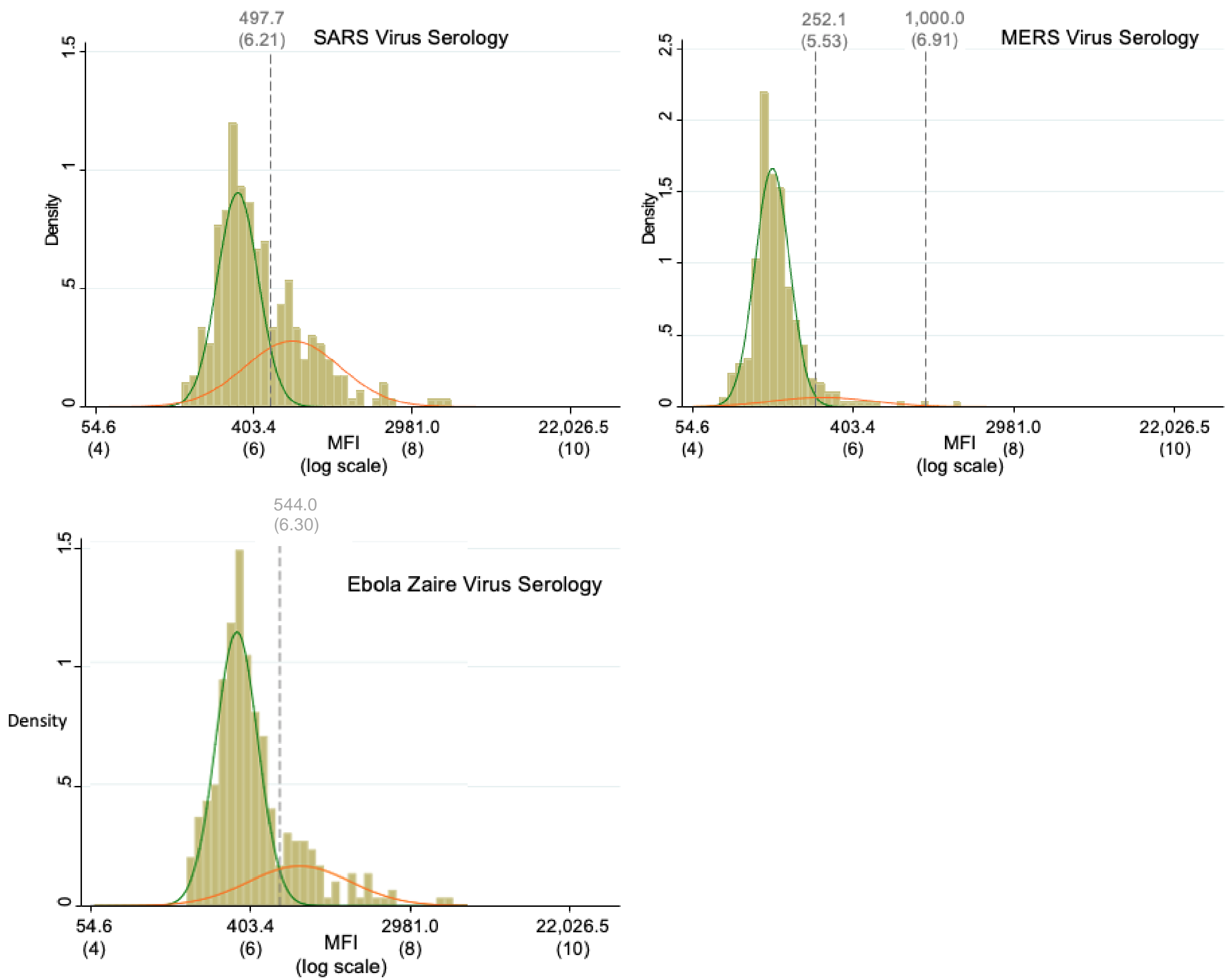

Fig 5.1: Density histogram and overlaid mixture of modelled distributions.

Density histograms (bars) estimated gaussian mixture model components (lines) for MFI and the natural log MFI for SARS-CoV-1 and MERS-CoV serological responses. For SARS-CoV-1 and Ebola Zaire serology, the threshold (dashed vertical line) corresponds to the intersection between the pair of predicted distributions. The threshold for SARS-CoV-1 serology and Ebola Zaire serology was determined as the natural antilog of 6.21 (MFI 497.7) and 6.30 (MFI 544.0) respectively. Finite mixture modelling was run in the statistical package Stata v15.1 (College Station, Texas, USA). An analytical threshold (dashed vertical line) for MERS-CoV was set at 1,000 MFI (natural antilog 6.91).

5.4.2 Hurdle modelling of seropositive animals and antibody levels

Results from binomial component of the fitted hurdle models indicated that seropositivity to Ebola Zaire and SARS-CoV-1 was not significantly associated with any of the measured individual-level covariates. Using the antibody level component of the hurdle models, Ebola Zaire antibody levels were significantly lower overall at the final catching session in February 2018 (p = 0.018) and SARS-CoV-1 antibody levels were significantly lower in non-pregnant seropositive females (p = 0.011) and at the second catching session in February 2016 (p= 0.038) and fifth catching session in August 2017 (p = 0.006) (Table 5.2 and Fig 5.2).

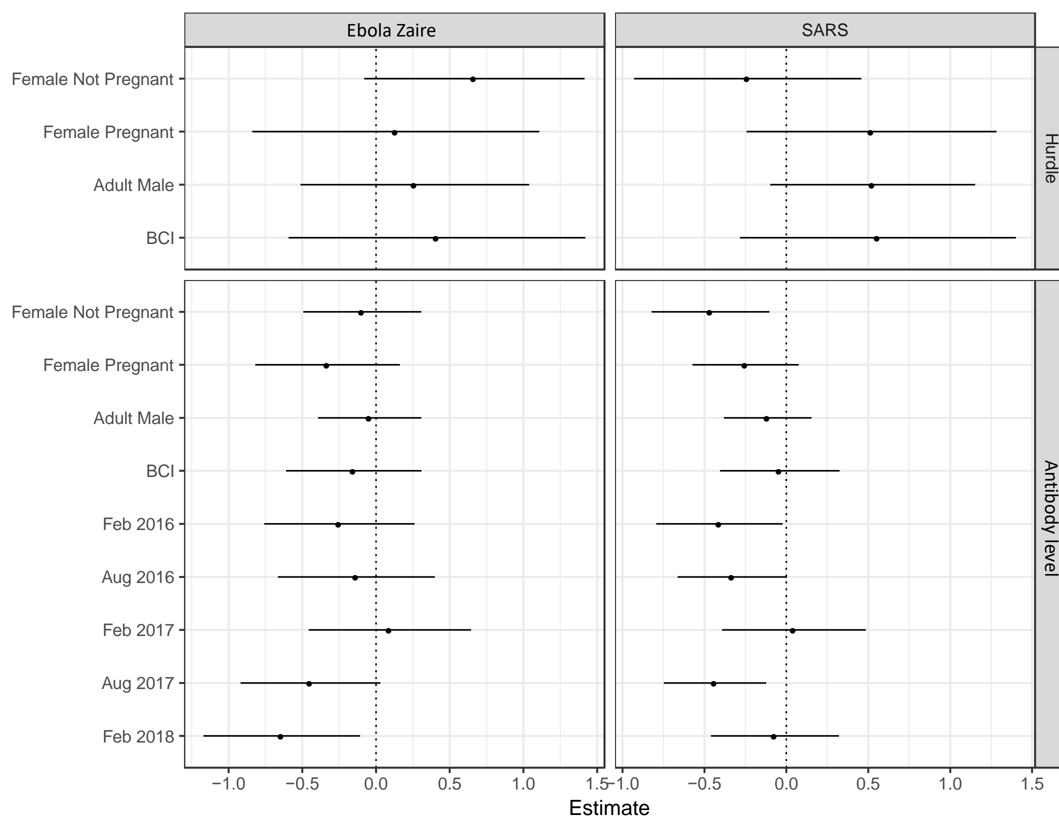

Fig 5.2: Effect plots for hurdle and antibody level models. Effects plots (estimates ± 95 % confidence intervals) from the negative binomial hurdle models for Ebola Zaire virus and SARS virus serology for Grey-headed flying foxes sampled between August 2015 and February 2018.

Table 5.2: Statistics for the hurdle and antibody level models

Odds ratios (OR), estimates, standard errors (SE), Z values and *p* values for negative binomial hurdle and antibody levels models for Ebola Zaire and SARS serology for Grey-headed flying foxes sampled between August 2015 and February 2018. Reference values relate to subadults (both male and female) for the hurdle and antibody levels model and for the first catching session (August 2015) for the antibody levels model. Preg = pregnant. BCI centred = Body condition index centred around the mean values. CI = confidence interval.

MODEL	Ebola Zaire					SARS				
HURDLE	Estimate	OR (95% CI)	SE	Z value	*p* value	Estimate	OR (95% CI)	SE	Z value	*p* value
Intercept	-1.720	-	0.204	-1.526	3.250	-0.970	-	0.221	-4.393	<0.001
Adult male	0.263	1.30	0.308	-0.118	0.507	0.526	1.69	0.319	1.650	0.098
Female Not Preg	0.667	1.94	0.313	0.127	0.080	-0.236	-1.26	0.353	-0.667	0.504
Female Preg	0.135	1.14	0.379	0.151	0.786	0.520	1.68	0.389	1.335	0.181
BCI centred	0.413	1.51	0.416	3.126	0.421	0.560	1.75	0.429	1.300	0.193

ANTIBODY LEVEL	Estimate	SE	Z value	*p* value	Estimate	SE	Z value	*p* value
Intercept	7.290	0.206	35.322	2.649	7.250	0.144	50.365	1.113
Adult male	-0.043	0.178	-0.241	0.809	0.099	0.137	-0.830	0.406
Female Not Preg	-0.094	0.204	-0.460	0.646	**0.505**	**0.184**	**-2.520**	**0.011**
Female Preg	-0.329	0.250	-1.313	0.189	0.181	0.166	-1.500	0.133
BCI centred	-0.150	0.235	-0.644	0.520	0.193	0.186	-0.215	0.829
Feb-16	-0.249	0.260	-0.953	0.340	**-0.408**	**0.197**	**-0.207**	**0.038**
Aug-16	-0.133	0.271	-0.489	0.625	-0.330	0.170	-1.939	0.052
Feb-17	0.094	0.281	-0.335	0.737	0.046	0.224	0.207	0.836
Aug-17	-0.444	0.242	-1.835	0.066	**-0.436**	**0.160**	**-2.732**	**0.006**
Feb-18	**-0.639**	**0.271**	**-2.360**	**0.018**	-0.070	0.199	-0.351	0.725

5.4.3 Recapture seroprevalence analysis

Over the six sampling sessions, four bats were recaught - three males and one female (Table 5.3). No changes to serostatus in these individuals were seen for Ebola Zaire virus across catching sessions. However, one male individual transitioned from seropositive to seronegative over 6 months for SARS-CoV-1 between September 2015 and February 2016, and another became (marginally) seropositive over 12 months between August 2016 and August 2017.

Table 5.3: Seroprevalence changes in recaptured Grey-headed flying foxes

The sex, weight (Wt), body condition index (BCI) and study identification number (Bat ID) of four Grey-headed flying foxes recaptured between August 2015 and August 2017 at the Adelaide Camp, Adelaide, South Australia and their median fluorescence intensity (MFI) serostatus for Ebola Zaire virus, SARS-CoV-1 and MERS-CoV. M= Male; F=Female.

Bat Project ID	Date of capture	Sex	Wt / g	BCI	Ebola Zaire MFI	Ebola Zaire positive	SARS-CoV-1 MFI	SARS-CoV-1 positive	MERS-CoV MFI	MERS-CoV positive
6 & 72	31 Aug 2015	F	844	29.8	259	-	205	-	140	-
	26 Feb 2016		763	26.9	328	-	348	-	118	-
23 & 70	2 Sept 2015	M	820	30.1	222	-	**1174**	+	172	-
	26 Feb 2016		744	26.6	358	-	208	-	94	-
46 & 81	3 Sept 2015	M	666	25.4	396	-	281	-	121	-
	26 Feb 2016		773	28.0	389	-	309	-	154	-
145 & 252	10 Aug 2016	M	818	30.7	301	-	407	-	337	-
	13 Aug 2017		830	30.8	320	-	591	+	321	-

5.5 Discussion

Our study indicates that the Grey-headed flying foxes in Adelaide show significant exposure to protein antigens of Ebola Zaire virus and SARS-CoV-1. Overall true seroprevalence to protein antigens to Ebola Zaire virus and SARS-CoV-1 was estimated to be 26.7% (CI: 19.9%-34.4%) and 42.5% (CI: 34.3%-51.2%), respectively. These results indicate that this population of flying foxes has been exposed to both Ebola Zaire-like and SARS-like viruses that cross-react with the Ebola Zaire and SARS viral antigens used in this study. Seroprevalence to these two viruses has not hitherto been reported from Australia.

In contrast, no substantial evidence of exposure to MERS-CoV was detected. Based on the determination of exposure sub-populations using finite mixture modelling, seroprevalence to MERS-CoV was considered negative, however if an analytical cut off > 1000 MFI is used, then two individuals were marginally positive (MFI 1018 and 1444). To date, no betaCoVs from the sub-genus merbecovirus (within which MERS-CoV belongs) have been detected in fruit bats (Wong et al, 2019). This may be the reason why there was no evidence of MERS-CoV seroprevalence in the Grey-headed flying fox camp in Adelaide.

The seroprevalence of Ebola Zaire virus in the Grey-headed flying fox camp using our Luminex multiplex binding assay is much higher than reported using ELISA IgG in bat species from Congo (5%) (Pourrut et al, 2007), Gabon (4%) (Pourrut et al, 2009) and Zambia (9%) (Ogawa et al, 2015), and using ELISA IgG and Western blots in fruit bats from Bangladesh (3.5%) (Olival et al, 2013). However, our seroprevalence estimate is lower than that reported using ELISA IgG in four bat species in Ghana (36.3%) (Hayman at al, 2012). Using a similar multiplex assay to our study, two large studies detected Ebola virus antibodies in six fruit bat and one insectivorous bat species (De Nys et al, 2018) and primates (Ayouba et al, 2019) in Central Africa. A further study in pteropodid fruit bats in Singapore detected antigen proteins to Bundibugyo, Ebola and Sudan viruses indicating filovirus

circulation among three bat species widely distributed throughout South East Asia (Laing et al, 2018).

Coronavirus antibodies have been detected in twenty-three species of bats from East Timor, Indonesia, Malaysia, and Papua New Guinea across the microbat genera *Miniopterus*, *Myotis*, *Rhinolophus*, *Vespadelus* as well as the Black flying fox in Australia (Smith et al., 2016). Luminex multiplex antibody assays have also been used to detect coronaviruses in camels (Crameri et al, 2015) and humans (Trivedi et al, 2018). The phylogeography of bat coronaviruses suggests that bat SARS-like coronaviruses form a monophyletic clade that is both phylogenetically distinct from other bat coronaviruses and geographically isolated (Cui et al, 2007). Ecological and epidemiologic analyses show that patterns of coronavirus diversity correlate with those of bat diversity (Anthony et al, 2017). Furthermore, co-phylogenetic reconciliation analysis showed that host switching has contributed to coronavirus evolution, and a preliminary analysis suggests that regional variation exists in the dynamics of this process (Anthony et al, 2017). Our results raise questions about the origins of the filovirus and coronavirus to which the Adelaide camp has been exposed, and how and when this might have occurred. It is conceivable that an Ebola Zaire-like virus and a SARS-CoV-1-virus coevolved with bats and have been circulating in the Grey-headed flying fox population or sympatric populations historically, or that infection has occurred relatively recently, or that these viruses resulted from recombination events relatively recently.

In 2016, a novel coronavirus, Ro-Bat CoV GCCDC1, was discovered in the Dawn bat (*Eonycteris spelaea*), a member of the Pteropodid family from South-East Asia (Mendenhall et al, 2016). This species also had an Ebola virus seroprevalence of 9.1 % (Laing et al, 2018) and is sympatric with the Black flying fox (*Pteropus alecto*) on Sumba Island in Indonesia. The distribution of the Grey-headed flying foxes overlaps considerably with that of the Black flying fox. Consequently, it is conceivable

that circulation of and exposure to coronaviruses could occur across the multi-bat species distribution range continuum from Southeast Asia to Queensland through to South Australia.

We found that SARS-CoV-1 antibody levels were significantly lower in non-pregnant seropositive females. Although the reason for this result is unknown, it is possible that sexual maturity, sexual activity, and/or pregnancy contribute to the infection dynamics of SARS-CoV-1 in this population. During our study, the SARS-CoV-1 antibody levels of one male switched from distinctively seropositive to seronegative over a six-month period between September 2015 and February 2016. This suggests waning immunity to SARS-CoV-1 and no viral exposure over that period. However, more frequent serological testing could improve our understanding of the periodicity of infection and longevity of antibody presence. Unfortunately, it was not possible to evaluate viral presence particularly in urine, faeces and oral swabs and to compare results with seroprevalence status. In the future, longitudinal sampling coupled with viral detection could allow estimation of viral sequence diversity which may help to elucidate transmission pathways (Plowright et al, 2016). However, in this study we used bi-annual sampling to minimise capture stress and potential mismothering during October to December and mating disruption in April to May.

Conclusion

Our results suggest an uncharacterised bat-borne filovirus and coronavirus that are antigenically related to Ebola Zaire virus and SARS virus are circulating in the Grey-headed flying fox population in Adelaide. In turn, this suggests this species may contribute a role in cross-species filovirus and coronavirus infection dynamics across fruit bats in Australia and Asia. This corroborates previous evidence which suggests that filoviruses and coronaviruses are harboured across a much larger geographic range than previously assumed (Negredo et al, 2011). To date, these viruses may have remained undetected due to their inability to cross the species barrier, the rarity of spillovers into humans or domestic animals, or the fact that spillover events cause mild or no disease (Laing et al,

2018). The zoonotic potential of filoviruses (like Ebola Zaire virus) and beta coronaviruses (like SARS-CoV-1 and MERS-CoV) should not be underestimated and comprehensive surveillance of pteropodids and other bat species, including serology and detection of viral nucleic acid, along with virus isolation, would help elucidate the ecology of these viruses in Australia and South East Asia and identify which bat species function as maintenance or reservoir hosts. Furthermore, we concur with Anthony et al, (2017) in that our study attempts to advance our understanding of the natural history of viruses, not to create alarm for humans or to instigate reactive dispersal of bats. Indeed, such dispersals may have unanticipated consequences, possibly even boosting viral shedding (Streicker et al, 2012). We highlight the need to consider any public health risk analysis carefully and until further research has been undertaken, we suggest high standards of biosecurity are warranted when close human-flying fox contact occurs.

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5.7 References

Andersen KG, Rambaut A, Lipkin WI, Holmes EC and Garry RF. (2020). The proximal origin of SARS-CoV-2. *Nature Medicine*, 26, 450–452. doi: 10.1038/s41591-020-0820-9

Anthony SJ, Johnson CK, Greig DJ, Kramer S, Che X, Wells H, Hicks AL, Joly DO, Wolfe ND, Daszak P, Karesh W, Lipkin WI, Morse SS, PREDICT Consortium, Mazet JAK, Goldstein T. (2017). Global patterns in coronavirus diversity. *Virus Evolution*, 3:1. doi: 10.1093/ve/vex012.

Ayouba A, Ahuka-Mundeke S, Butel C, Kingebeni MP, Loul S, Tagg N, Villabona-Arenas C-J, Lacroix A, Dimbo-Kumogo S-P, Keita AK, Toure A, Couacy-Hymann E, Calvignanc-Spencer S, Leendertz FH, Formenty P, Delaporte E, Muyembe-Tamfum J-J, Ngole EM, Peeters M. (2019). Extensive serological survey of multiple African nonhuman primate species reveals low prevalence of immunoglobulin G antibodies to 4 Ebola virus species. *Journal of Infectious Disease*, 220(10):1599-1608. doi: 10.1093/infdis/jiz006

Banerjee A, Kulcsar K, Misra V, Frieman M, Mossman K. (2019). Bats and Coronaviruses. *Viruses*, 11:41. doi: org/10.3390/v11010041

Barr JA, Smith C, Marsh GA, Field H, Wang L-F. (2012). Evidence of bat origin for Menangle virus, a zoonotic paramyxovirus first isolated from diseased pigs. *Journal of General Virology,* 93, 2590–2594. doi 10.1099/vir.0.045385-0

Bempong NF, De Castañeda RR, Schütte S, Bolon I, Keiser O, Escher G, Flahault A. (2019). Precision Global Health - The case of Ebola: a scoping review. *Journal of Global Health,* 9:1. doi: 10.7189/jogh.09.010404

Boardman WSJ, Baker ML, Boyd V, Crameri G, Peck GR, Reardon T, Smith IG, Caraguel CGB, Prowse TAA. (2020). Seroprevalence of three paramyxoviruses; Hendra virus, Tioman virus, Cedar virus and a rhabdovirus, Australian bat lyssavirus, in a range expanding fruit bat, the Grey-headed flying fox (*Pteropus poliocephalus*). *PLOS One*. doi: 10.1371/journal.pone.0232339

Bossart KN, McEachern JA, Hickey AC, Choudhry V, Dimitrov DS, Eaton BT, Wang L-F. (2007). Neutralization assays for differential henipavirus serology using Bio-Plex Protein array systems. *Journal of Virological Methods,* 142: 29–40. doi: 10.1016/j.jviromet.2007.01.003.

Boyd V, Smith I, Crameri G, Burroughs AL, Durr PA, White J, Cowled C, Marsh GA, Wang L-F. (2015). Development of multiplexed bead arrays for the simultaneous detection of nucleic acid from multiple viruses in bat samples. *Journal of Virological Methods*, 223: 5-12. doi: 10.1016/j.jviromet.2015.07.004

Breed AC, Yu M, Barr JA, Crameri G, Thalmann CM, Wang LF. (2010). Prevalence of henipavirus and rubulavirus antibodies in pteropid bats, Papua New Guinea. *Emerging Infectious Disease,* 16: 1997–1999. doi: 10.3201/eid1612.100879

Breed AC, Breed MF, Meers J, Field HE. (2011). Evidence of Endemic Hendra Virus Infection in flying foxes (*Pteropus conspicillatus*)—Implications for Disease Risk Management. *PLOS ONE*, 6 (12). doi: 10.1371/journal.pone.0028816

Burroughs AL, Durr PA, Boyd V, Graham K, White JR, Todd S, Barr J, Smith I, Baverstock G, Meers J, Crameri G, Wang L-F. (2016). Hendra virus infection dynamics in the Grey-headed flying fox (*Pteropus poliocephalus*) at the southern-most extent of Its range: further evidence this species does not readily transmit the virus to horses. *PLOS ONE*, 11 (6) doi: 10.1371/journal.pone.0155252

Carlson CJ, Albery GF, Merow C, Trisos CH, Zipfel CM, Eskew EA, Olival KJ, Ross N, Bansal, S. (2020). Climate change will drive novel cross-species viral transmission. *Pre print bioRxiv*. doi: 10.1101/2020.01.24.918755

Chen J, Rossiter SJ, Flanders JR, Sun YH, Hua PY, Miller-Butterworth C, Lui X, Rajan KE, Zhang S. (2010). Contrasting genetic structure in two co-distributed species of Old World fruit bat. *PLOS ONE,* 5:e13903. doi: 10.1371/journal.pone.0013903

Crameri G, Durr PA, Barr J, Yu M, Graham K, Williams OJ, Kayali G, Smith D, Peiris M, MacKenzie JS, Wang L-F (2015). Absence of MERS-CoV antibodies in feral camels in Australia: implications for the pathogen's origin and spread. *One Health*, 1, 76–82. doi: 10.1016/j.onehlt.2015.10.003

Cui J, Han N, Streicker D, Li G, Tang X, Shi Z, Hu Z, Zhao G, Fontanet A, Guan Y, Wang L-F, Jones G, Field HE, Daszak P, Zhang S. (2007). Evolutionary relationships between bat coronaviruses and their hosts. *Emerging Infectious Disease,* 13:1526–1532. doi: 10.3201/eid1310.070448

de Groot RJ, Baker SC, Baric RS, Brown CS, Drosten C, Enjuanes L, Fouchier RA, Galiano M, Gorbalenya AE, Memish ZA, Perlman S, Poon LL, Snijder EJ, Stephens GM, Woo PC, Zaki AM, Zambon M, Ziebuhr J. (2013) Middle east respiratory syndrome coronavirus (MERS-CoV): announcement of the coronavirus study group. *Journal of Virology*, 87:7790–7792. doi: 10.1128/JVI.01244-13

De Nys HM, Kingebeni P, Keita AK, Butel C, Thaurignac G, Villabona-Arenas C, and Peeters, M. (2018). Survey of Ebola Viruses in Frugivorous and Insectivorous Bats in Guinea, Cameroon, and the Democratic Republic of the Congo, 2015–2017. *Emerging Infectious Diseases*, *24*(12), 2228-2240. doi: 10.3201/eid2412.180740.

Dohoo IR, Martin SW, Stryhn H. (2009). Screening and diagnostic tests. In Veterinary Epidemiologic Research, 2nd ed., pp.102, AVC Publishing, Charlottetown, Prince Edward Island, Canada

Drexler JF, Corman VM, Drosten C. (2014) Ecology, evolution and classification of bat coronaviruses in the aftermath of SARS. Antiviral Research 101:45–56. doi: 10.1016/j.antiviral.2013.10.013

Edson D, Field H, McMichael L, Vidgen M, Goldspink L, Broos A, Melville D, Kristoffersen J, de Jong C, McLaughlin A, Davis R, Kung N, Jordan D, Kirkland D, Smith C. (2015). Routes of Hendra virus excretion in naturally infected flying foxes: implications for viral transmission and spillover risk. *PLOS ONE*. 10 (10): e0140670. doi: 10.1371/journal.pone.0140670

Edson D, Peel AJ, Huth L, Mayer DG, Vidgen ME, McMichael L , Broos A, et al, Melville D, Kristoffersen J, de Jong C, McLaughlin A, Field HE. (2019). Time of year, age class and body condition predict Hendra virus infection in Australian black flying foxes (*Pteropus alecto*). *Epidemiology and Infection*, Volume 147, e240. doi: 10.1017/S0950268819001237.

Epitools website. (2020a). Estimated true prevalence. Available online: https://epitools.ausvet.com.au/trueprevalence (accessed on 29 December 2020).

Epitools website. (2020b). FreeCalc data analysis. Available online: https://epitools.ausvet.com.au/freecalcone (accessed on 29 December 2020).

Field HE, de Jong C, Melville D, Smith C, Smith I, Broos A, Kung YH, McLaughlin A, Zeddeman A. (2011). Hendra virus infection dynamics in Australian fruit bats. *PLOS ONE* 6, e28678. doi: 10.1371/journal.pone.0028678

Guan Y, Zheng BJ, He YQ, Liu XL, Zhuang ZX, Cheung CL, Luo SW, Li PH, Zhang LJ, Guan YJ, Butt KM, Wong KL, Chan KW, Lim W, Shortridge KF, Yuen KY, Peiris JS, and Poon LL. (2003). *Isolation and characterization of viruses related to SARS coronavirus from animals in southern China. Science*, 302: 276-*278.* doi: 10.1126/science.1087139

Hall LS and Richards G. (2000). Flying foxes: Fruit and Blossom Bats of Australia. University of New South Wales Press, Sydney

Hayman DTS, Yu M, Crameri G, Wang L-F, Suu-Ire R, Wood JLN, Cunningham AA. (2012). Ebola Virus Antibodies in Fruit Bats, Ghana, West Africa. *Emerging Infectious Diseases*, 18, 1207–1209. doi: 10.3201/eid1807.111654

He B, Feng Y, Zhang H, Xu L, Yang W, Zhang Y, Li X, Tu C. (2015). Filovirus RNA in fruit bats, China. *Emerging Infectious Diseases*, 21:1675–1677. doi: 10.3201/eid2109.150260

Holmes EC and Rambaut A. (2004). Viral evolution and the emergence of SARS coronavirus. *Philosophical Transactions Royal Society of London B: Biological Sciences*, 359, 1059–1065. doi: 10.1098/rstb.2004.1478.

Holz PH, Lumsden LF, Druce J, Legione AR, Vaz P, Devlin JM, Hufschmid J. (2018). Virus survey in populations of two subspecies of bent-winged bats (*Miniopterus orianae bassanii* and *oceanensis*) in south-eastern Australia reveals a high prevalence of diverse herpesviruses. *PLOS ONE,* 13(5): e0197625. doi: 10.1371/journal.pone.0197625

Hu B, Zeng LP, Yang XL, Ge XY, Zhang W, Li B, Xie J-Z, Shen Y-Z, Wang N, Luo D-S, Zheng X-S, Wang M-N, Daszak P, Wang L-F, Cui J, Shi Z-L. (2017). Discovery of a rich gene pool of bat SARS-related

coronaviruses provides new insights into the origin of SARS coronavirus. *PLOS Pathogens*, 13. doi: 10.1371/journal.ppat.1006698

ICTV Taxonomy History: Cornidovirineae. Available online: https://talk.ictvonline.org/taxonomy/p/taxonomy-history?taxnode_id=20186105 (accessed on 1 December 2020).

Jeong J, Smith CS, Peel AJ, Plowright RK, Kerlin DH, McBroom J, McCallum H. (2017). *Epidemiology and Infection*, 145, 2053–2061. doi: 10.1017/S0950268817000991

Jonsson NN, Johnston SD, Field H, de Jong C, Smith C. (2004). Field anaesthesia of three Australian species of flying fox. *Veterinar Record,* 154:664. doi: 10.1136/vr.154.21.664

Laing ED, Mendenhall IH, Linster M, Low DHW, Chen Y, Yan, L, Sterling SL, Borthwick S, Neves, ES, Lim, JSL, Skiles M, Lee BPYH, Wang L_F, Broder CC, Smith GJD. (2018). Serologic evidence of fruit bat exposure to filoviruses, Singapore, 2011-2016. *Emerging Infectious Diseases,* 24:1 122-126. doi: 10.3201/eid2401.170401

Latinne A, Hu B, Olival KJ, Zhu G, Zhang L, Li H, Chmura AA, Field HE, Zambran-Torrelio C, Epstein JH, Li B, Zhang W, Wang L-F, Shi Z-L, Daszak P. (2020). Origin and cross-species transmission of bat coronaviruses in China. *Nature Communications,* 11: 4235. doi: 10.1038/s41467-020-17687-3

Lau SKP, Li KSM, Huang Y, Shek CT, Tse H, Wang M , Choi GKY, Xu H, Lam CSF, Guo R, Chan, K-H, Zheng B-J, Woo PCY, Yuen K-Y. (2010). Ecoepidemiology and complete genome comparison of different strains of Severe Acute Respiratory Syndrome-related Rhinolophus bat coronavirus in China

reveal bats as a reservoir for acute, self-limiting infection that allows recombination events. *Journal of Virology*, 84, 2808–19. doi: 10.1128/JVI.02219-09

Lau SK, Zhang L, Luk HKH, Xiong L, Peng X, Li KSM, He X, Zhao PS-H, Fan RYY, Wong ACP, Ahmed SS, Cai J-P, Chane JFW, Sun Y, Jin D, Chen H, Lau TCK, Kok RKH, Yuen K-Y, Woo PCY. (2018). Receptor usage of a novel bat lineage C betacoronavirus reveals evolution of MERS-related coronavirus spike proteins for human DPP4 binding. *Journal of Infectious Diseases*, 218 (2), 197-207. doi: 10.1093/infdis/jiy018

Leopardi S, Holmes EC, Gastaldelli M, Tassoni L, Priori, P, Scaravelli D, Zamperin G, De Benedictis P. (2018). Interplay between co-divergence and cross-species transmission in the evolutionary history of bat coronaviruses. *Infection, Genetics and Evolution,* 58 279-289. doi: 10.1016/j.meegid.2018.01.012

Leroy EM, Kumulungui B, Pourrut X, Rouquet P, Hassanin A, Yaba P, Delicat A, Paweska JT, Gonzalez JP and Swanepoel R. (2005). Fruit bats as reservoirs of Ebola virus. *Nature,* 438; 575–576. doi: 10.1038/438575a

Li W, Sh,i Z., Yu M, Ren W, Smith C, Epstein JH, Wang H, Crameri G, Hu Z, Zhang J, McEachern J, Field HE, Daszak P, Eaton BT, Zhang S, Wang L-F. (2005). Bats are natural reservoirs of SARS-like coronaviruses. *Science*, 310 (5748): 676-679. doi: 10.1126/science.1118391.

McNabb L, Barr J, Crameri G, Jusva S, Colling A, Riddell S, Colling A, Boyd V, Broder CC, Wang L-F, Lunt R. (2014). Henipavirus microsphere immune-assays for detection of antibodies against Hendra virus. *Journal of Virological Methods*, 200: 22-28. doi: 10.1016/j.jviromet.2014.01.010

Mendenhall IH, Borthwick S, Neves ES, Low D, Linster M, Liang B, Skiles M, Jayakuma J, Han H, Gunalan V, Lee BPY-H, Okahara K,Wang L-F, Maurer-Stroh S, Su YCF, Smith GJD. (2016). Identification of a lineage D betacoronavirus in cave nectar bats (*Eonycteris spelaea*) in Singapore and an overview of lineage D reservoir ecology in SE Asian bats. *Transboundary and Emerging Diseases*, 64, 1790-1800. doi: 10.1111/tbed.12568

Memish ZA, Mishra N, Olival KJ, Fagbo SF, Kapoor V, Epstein JH, Alhakeem R, Durosinloun A, Al Asmari M, Islam A, Kapoor A, Briese T, Daszak P, Al Rabeeah AA, Lipkin WI. (2013). Middle East respiratory syndrome coronavirus in bats, Saudi Arabia. *Emerging Infectious Diseases*, 19, 1819–1823. doi: 10.3201/eid1911.131172

Negredo A, Palacios G, Vazquez-Moron S, Gonzalez F, Dopazo H, Molero F. (2011). Discovery of an Ebolavirus-like filovirus in Europe. *PLOS Pathogens,* 7:e1002304. doi: 10.1371/journal.ppat.1002304

Ogawa H, Miyamoto H, Nakayama E, Yoshida R, Nakamura I, Sawa H, Shii A, Thomas Y, Nakagawa E, Matsuno K ... Takada A. (2015). Seroepidemiological prevalence of multiple species of filoviruses in fruit bats (*Eidolon helvum*) migrating in Africa. *Journal of Infectious Disease*, 212(Suppl 2): S101–8. doi: 10.1093/infdis/jiv063

Olival KJ, Islam A, Yu M, Anthony SJ, Epstein JH, Khan SA, Khan SU, Crameri G, Wang L-F, Lipkin WI, Luby SP, Daszak P. (2013). Ebola virus antibodies in fruit bats, Bangladesh. *Emerging Infectious Diseases*, 19, 270–273. doi: 10.3201/eid1902.120524

Olival, K.J and Hayman DTS. (2014). Filoviruses in Bats: Current Knowledge and Future Directions. *Viruses,* 6, 1759-1788. doi:10.3390/v6041759

Peel AJ, Bake KS, Crameri G, Barr JA, Hayman DTS, Wright E, Broder CC, Fernandez-Loras A, Fooks AR, Wang L-F, Cunningham AA, Wood JLN. (2012). Henipavirus neutralising antibodies in an isolated island population of African fruit bats. *PLOS ONE, 7* (1): e30346. doi: 10.1371/journal.pone.0030346

Peel AJ, McKinley TJ, Baker KS, Barr JA, Crameri G, Hayman DTS, Feng Y-R, Broder CC, Wang L-F, Cunningham AA,Wood JLN. (2013). Use of cross-reactive serological assays for detecting novel pathogens in wildlife: Assessing an appropriate cutoff for henipavirus assays in African bats. *Journal of Virological Methods*, 193: 295–303. doi: 10.1016/j.jviromet.2013.06.030

Philbey AW, Kirkland PD, Ross AD, Field HE, Srivastava M, Davis RJ, Love RJ. (2008). Infection with Menangle virus in flying foxes (Pteropus spp.) in Australia. *Australian Veterinary Journal*, 86: 449–454. doi: 10.1111/j.1751-0813.2008.00361.x

Plowright RK, Peel AJ, Streicker DG, Gilbert AT, McCallum H, Wood J, Baker ML, Restif O. (2016). Transmission or Within-Host Dynamics Driving Pulses of Zoonotic Viruses in Reservoir-Host Populations. *PLOS Neglected Tropical Diseases*, 10 (8). doi: 10.1371/journal.pntd.0004796

Pourrut X, Delicat A, Rollin PE, Ksiazek TG, Gonzalez JP, Leroy EM. (2007). Spatial and temporal patterns of Zaire ebolavirus antibody prevalence in the possible reservoir bat species. *Journal of Infectious Disease*, 196 S176-S183. doi: 10.1086/520541

Pourrut X, Souris M, Towner JS, Rollin PE, Nichol ST, Gonzalez JP, Leroy E. (2009). Large serological survey showing cocirculation of Ebola and Marburg viruses in Gabonese bat populations, and a high seroprevalence of both viruses in *Rousettus aegyptiacus*. *BMC Infectious Diseases*, 9. doi:10.1186/1471-2334-9-159.

Sanchez A, Khan AS, ZakiS R, Nabel GJ, Ksiazek T, Peters C. (2001). Filoviridae: Marburg and Ebola viruses. In: Knipe DM, Howley PM, editors. Fields virology, 4th ed. Philadelphia: Lippincott Williams & Wilkins; p. 1279–304

Smith CS, de Jong CE, Meers, J, Henning, J, Wang-L-F, Field HE. (2016). Coronavirus Infection and Diversity in Bats in the Australasian Region. *EcoHealth,* 13, 72–82: doi: 10.1007/s10393-016-1116-x

Streicker DG, Recuenco S, Valderrama W, Benavides JG, Vargas I, Pacheco V, Condori Condori RE, Montgomery J, Rupprecht CE, Rohani P, Altizer S. (2012). Ecological and anthropogenic drivers of rabies exposure in vampire bats: implications for transmission and control. *Philosophical Transactions Royal Society of London B: Biological Sciences*, 279, 3384–3392. doi:10.1098/rspb.2012.0538

Trivedi SU, Miao C, Sanchez JE, Caidi H, Tamin A, Haynes L, Thornburg NJ. (2019). Development and evaluation of a Multiplexed Immunoassay for simultaneous Detection of serum IgG Antibodies to six Human Coronaviruses. *Nature Scientific Reports,* 9: 1390. doi: 10.1038/s41598-018-37747-5

Wang L-F and Anderson DE. (2019). Viruses in bats and potential spillover to animals and humans. *Current Opinion in Virology*, 34:79–89. doi: 10.1016/j.coviro.2018.12.007

Wong ACP, Li X, Lau SKP, Woo PCY. (2019). Global Epidemiology of Bat Coronaviruses. *Viruses*, 11, 174. doi:10.3390/v11020174

Woo PC, Huang Y, Lau SK, Yuen KY. (2010). Coronavirus genomics and bioinformatics analysis. *Viruses*, 2: 1804–1820. doi: 10.3390/v2081803

Yang XL, Zhang YZ, Jiang RD, Guo H, Zhang W, Li B, Wang N, Wang L, Waruhiu C, Zhou JH, Li SY, Daszak P, Wang LF, Shi ZL. (2017). Genetically diverse filoviruses in Rousettus and Eonycteris spp. bats, China, 2009 and 2015. *Emerging Infectious Disease,* 23: 482–486. doi: /10.3201/eid2303.161119

Yu M, Stevens V, Berry JD, Crameri G, McEachern JA, Tu C, Shi Z, Lian G, Weingarti H, Cardosa J, Eaton BT, Wang, L-F. (2008). Determination and application of immunodominant regions of SARS coronavirus spike and nucleocapsid proteins recognized by sera from different animal species. *Journal of Immunological Methods,* 331, 3311–12. doi: 10.1016/j.jim.2007.11.009

Yuan JF, Zhang YJ, Li JL, Zhang YZ, Wang LF, Shi ZL. (2012). Serological evidence of Ebolavirus infection in bats. *China Virology Journal,* 9:236. doi: 10.1186/1743-422X-9-236

Zhang C, Wang Z, Cai J, Yan X, Zhang F, Wu J, Xu L, Zhao Z, Hu T, Tu C, He B. (2020). Seroreactive Profiling of Filoviruses in Chinese Bats Reveals Extensive Infection of Diverse Viruses. *Journal of Virology,* 94 (7) e02042-19; doi: 10.1128/JVI.02042-19

Chapter 6: Discussion

In 2011, several hundred individuals of the nationally threatened Grey-headed flying fox formed a camp in Adelaide's Botanic Park, expanding on the species' previous distribution range across Victoria, NSW and Queensland. Since then, the size of the camp has grown considerably, and it is now a camp of 'national significance' (Referral guideline, 2015). But why did this species expand its range into South Australia and how did it establish in this new location so successfully? Because they are volant, bats are easily capable of 'jump dispersal' into new areas. However, it has been difficult to disentangle the relative contribution of 'pull' factors drawing flying foxes into South Australia and Adelaide and 'push' factors driving the flying foxes to leave their normal habitats.

A variety of 'push' factors have been previously attributed to flying fox range expansions, including a reduction in resources (Tidemann 1999; van der Ree et al, 2006), direct competition with other flying fox species (Webb and Tidemann 1995) and anthropogenic alteration of ecosystems (Ancilloto et al, 2016) including human-induced climate change (Parris and Hazell 2005, Bradshaw et al, 2006). However, 'pull' factors associated with immigration into the Adelaide region and the establishment of a permanent camp have remained poorly understood. The first purpose of my work was to shed light on some of the factors that enabled the permanent establishment of a camp in Adelaide, in particular the sourcing of sustainable food resources by Grey-headed flying foxes in this new environment. Furthermore, the range expansion of any species can have consequences for biodiversity and humans within the newly occupied regions. In the case of flying foxes, in general, as they have become more urbanised, they can have impacts on amenities including plane strikes, power outages and commercial fruit consumption all of which can bring them into conflict with humans. Of concern is the additional perceived risk to public health through their carriage of potentially zoonotic viruses. The second purpose of my work was, therefore, to investigate the extent of the overlap of the space use as well as to assess the bio-hazard potential of the camp.

6.1. Aim 1. To characterise the foraging resources and habits of the Adelaide's Grey-headed flying foxes that supported the establishment of a permanent camp

We showed that, during spring, the flying foxes remained within Adelaide's residential area and foraged rarely beyond the city's boundaries (**Chapter 2**). Ground-truthing revealed that flying foxes were eating plants that are not native to South Australia (but are found elsewhere in Australia), as well as plants that are exotic to Australia. All of these foraging resources were found on streets, parks and in private residencies. This was further confirmed by metabarcoding of plant DNA with flying fox faeces (**Chapter 3**), with over 81% of DNA sequences identified belonging to plants that are alien to South Australia. Taken together, these results suggest that Grey-headed flying foxes have been able to persist in Adelaide as a result of human-modified changes to the landscape. Individuals visited the same urban sites over several days which suggests they were finding abundant food resources during Spring 2015. This conclusion was further supported by the fact that Adelaide's flying foxes were in significantly better condition in winter than in summer **(Chapter 4)**, in contrast to results from NSW and Queensland**.** It appears that food resources, mainly planted by humans after European settlement, could be acting as a 'pull factor' to encourage flying foxes to visit and ultimately stay in Adelaide. Other interesting behavioural findings **(Chapter 2)** included: movements across Adelaide airport at night which may pose a risk to aircraft which may increase as the population increases; and the use of many water sources across Adelaide most likely so they can dip for cooling and hydration, but which could potentially lead to environmental contamination with pathogens with zoonotic potential.

6.2 Aim 2: To investigate the potential public health threats associated with the establishment of a permanent Grey-headed flying fox population in the Adelaide area.

Grey-headed flying foxes, like other flying foxes and bats in general, are known to be vectors of viruses with zoonotic potential. In Australia, Hendra virus and Australian bat lyssavirus have emerged as new zoonotic viruses of public health significance. In **Chapter 4**, we estimated the seroprevalence of Hendra virus in Grey-headed flying foxes in Adelaide was over 43%, which was similar to other studies in flying foxes in Australia (Plowright et al, 2008; Breed et al, 2011; Burroughs et al, 2016). Antibody levels of seropositive individuals were significantly associated with late pregnancy, as seen in flying foxes in other studies (Plowright et al, 2008; Breed et al, 2011). Since the inception of our study, Edson et al, (2015) discovered the main species shedding Hendra virus in urine was the Black flying fox, while noting no shedding in 2958 samples from 1168 Grey-headed flying foxes. Consequently, this species has been considered less important epidemiologically than the Black flying fox in Hendra virus infection dynamics. Although the Grey-headed flying fox population shows significant evidence of exposure to Hendra virus, the risk to public and animal health is more difficult to assess. Unexpectedly, we found there was no evidence of Australian bat lyssavirus seroconversion over our study period despite a previous finding of a positive diagnosis in a Grey-headed flying fox in the Adelaide camp in September 2012 (Cox-Witton, 2019). Our results suggest that either Australian bat lyssavirus was not circulating within the camp over the sampling period; that seropositivity is very short lived; or that infected flying foxes suffer rapid mortality and were thus not sampled at surveys.

Following on from this study, we showed in **Chapter 5** that 57 flying foxes were seropositive to protein antigens related to Ebola Zaire virus and 95 were seropositive to protein antigens related to Severe acute respiratory syndrome virus, which corresponded to an estimated true seroprevalence of 26.7% and 42.5% respectively. However, we found insufficient evidence of any exposure to

Middle Eastern Respiratory syndrome virus in the Adelaide camp. It is clear that an uncharacterised bat-borne filovirus and coronavirus that are antigenically related to Ebola Zaire virus and SARS virus are circulating in the Grey-headed flying fox population in Adelaide. In turn, this suggests this species may play a role in cross-species filovirus and coronavirus infection dynamics in fruit bats including flying foxes, across Australia and Asia. This substantiates previous evidence suggesting that filoviruses (Negredo et al, 2011) and coronaviruses are found across a much larger geographic range than previously assumed. This is not surprising considering the large and overlapping geographic distribution ranges of many bat species (Olival and Hayman, 2014).

In conclusion, we suggest Grey-headed flying foxes have been subject to mostly ‘pull factors’, specifically the foraging resources provided by alien plant species which have been introduced into the Adelaide region since European settlement, but we cannot discount ‘push factors’ caused by changes in the foraging resource availability in the rest of their distribution. These resources may have encouraged Grey-headed flying foxes to disperse to Adelaide and enabled them to establish and persist. This range expansion is associated with increased public health risk due to zoonotic viruses to which we now know these flying foxes have been exposed. We suggest high standards of biosecurity are warranted when humans handle flying foxes in Adelaide and elsewhere in Australia.

Our findings also highlight further questions to be considered by future research to deepen our understanding of Grey-headed flying fox ecology. Deploying GPS collars on more individuals over longer periods to further investigate fine-scale movements would provide more detailed information on landscape utilisation distribution and foraging preferences. This could help to clarify the risks to human amenities such as power outages and air transportation which could then be used to develop effective mitigation strategies and further clarify how foraging movements and activities have contributed to their persistence in Adelaide. For improved dietary understanding, random sampling of faeces collected weekly from beneath the camp over an annual cycle, together with fine tuning

methodologies to include barcodes such as *ITS2*, would allow for a more detailed characterisation of the seasonal variation in the species' diet and possibly provide improved taxonomic resolution of the foraging plants ingested. This would help to further clarify how foraging resources have contributed to their persistence in Adelaide. Developing a species distribution model for Grey-headed flying foxes could also provide information on the likelihood that the species will expand its range further into additional regions (e.g., south-east South Australia, Kangaroo Island and Tasmania) as the earth's climate changes. More comprehensive surveillance of individuals every three months including virus detection and isolation would help elucidate the ecology and source(s) of these viruses and add to the growing body of knowledge of the zoonotic risks posed by Australian flying foxes.

6.3 References

Ancillotto L, Santini L, Ranc N, Maiorano L, Russo D. (2016). Extraordinary range expansion in a common bat: The potential roles of climate change and urbanisation. *The Science of Nature*, 103:15. doi: 10.1007/s00114-016-1334-7.

Bradshaw WE and Holzapfel CM. (2006). Evolutionary response to rapid climate change. *Science*,312, 1477–1478. doi: 10.1126/science.1127000

Breed AC, Breed MF, Meers J, Field HE. (2011). Evidence of Endemic Hendra Virus Infection in flying foxes (*Pteropus conspicillatus*)—Implications for Disease Risk Management. *PLOS ONE*, 6 (12). doi: 10.1371/journal.pone.0028816

Cox-Witton K. Australian bat lyssavirus Bat Stats. Wildlife Health Australia website. Available from https://www.wildlifehealthaustralia.com.au/Portals/0/Documents/ProgramProjects/ABLV_Bat_Stats_Jun_2019.pdf. Accessed November 2019

Edson D, Field H, McMichael L, Vidgen M, Goldspink L, Broos A, Melville D, Kristoffersen J, de Jong C, McLaughlin A, Davis R, Kung N, Jordan D, Kirkland D, Smith C. (2015). Routes of Hendra virus excretion in naturally infected flying foxes: implications for viral transmission and spillover risk. *PLOS ONE*. 10 (10): e0140670. doi: 10.1371/journal.pone.0140670

Edson D, Peel AJ, Huth L, Mayer DG, Vidgen ME, McMichael L, Broos A, et al, Melville D, Kristoffersen J, de Jong C, McLaughlin A, Field HE. (2019). Time of year, age class and body condition predict Hendra virus infection in Australian black flying foxes (*Pteropus alecto*). *Epidemiology and Infection*, Volume 147, e240. doi: 10.1017/S0950268819001237.

Negredo A, Palacios G, Vazquez-Moron S, Gonzalez F, Dopazo H, Molero F. (2011). Discovery of an Ebolavirus-like filovirus in Europe. *PLOS Pathogens,* 7:e1002304. doi: 10.1371/journal.ppat.1002304

Olival KJ and Hayman DTS. (2014). Filoviruses in Bats: Current Knowledge and Future Directions. *Viruses*, 6, 1759-1788. doi:10.3390/v6041759

Parris K and Hazell D. (2005). Biotic effects of climate change in urban environments: The case of the Grey-headed flying fox (Pteropus poliocephalus) in Melbourne, Australia. *Biological Conservation* 124: 267–276. doi: 10.1016/j.biocon.2005.01.035

Plowright RK, Field HE, Smith C, Divljan A, Palmer C, Tabor G, Dazsak P, Foley JE. (2008). Reproduction and nutritional stress are risk factors for Hendra virus infection in Little red flying foxes (*Pteropus scapulatus*). *Proceedings of the Royal Society B: Biological Sciences*, 275: 861 – 869. doi: 10.1098/rspb.2007.1260

Referral guideline for management actions in Grey-headed and Spectacled flying fox camps, Commonwealth of Australia 2015. Available from http://www.environment.gov.au/system/files/resources/6d4f8ebc-f6a0-49e6-a6b6-82e9c8d55768/files/referral-guideline-flying fox-camps.pdf. In effect under the EPBC Act from 06-Dec-2001. Accessed 6 July 2020.

Tidemann CR. (1999). Biology and management of the Grey-headed flying fox, Pteropus poliocephalus. *Acta Chiropterologica*, 1: 151–164. doi: 10.7882/fs.2002.04

van der Ree R, McDonnell MJ, Temby ID, Nelson J and Whittingham E. (2006). The establishment and dynamics of a recently established urban camp of Pteropus poliocephalus outside their geographic range. *Journal of Zoology*, 268, 177–85. doi: 10.1017/S0952836901000486

Webb NJ and Tidemann CR. (1995). Hybridisation between Black (*Pteropus alecto*) and Grey-headed (*P. poliocephalus*) flying foxes (Megachiroptera: Pteropodidae). *Australian Mammalogy*,19-26.