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Revealing the virome of Sydney's red fox

Sarah J. Campbell

Department of Biological Sciences

Macquarie University, New South Wales, 2109

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Declaration

I declare that the work for this thesis has not been submitted for a higher degree to any other university or institution.

All research described in this thesis is my own original work.

Sarah J. Campbell

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Summary

Red foxes (*Vulpes vulpes*) are an invasive pest that contribute to the decline of native animals in Australia. Foxes are found in particularly high densities in cities, where the vast majority of Australia's human population resides. It is important to examine whether these urban-adapted pests are carriers of pathogens that may impact the health of humans and other animals. To understand the role that foxes may play as hosts of disease we used a meta-transcriptomic approach to describe the virome of the red fox. RNA from foxes and their ectoparasites were sequenced to reveal viromes of both urban and rural foxes from the Greater Sydney region. Foxes were found to harbour novel viruses including those from the *Astroviridae*, *Picobirnaviridae*, *Hepeviridae* and *Picornaviridae*. Rabbit haemorrhagic disease virus-2 was also found. Virome structure differed remarkably between urban and rural foxes, and rural foxes harboured a greater abundance of viruses compared to urban foxes. Overall, this study revealed Sydney's foxes are hosts to a wide diversity of viruses, some of which are close genetic relatives of viruses that infect domestic pets and humans. With continual rapid urbanisation, this finding highlights the need for research on wildlife diseases in urban landscapes.

Chapter I: Introduction

The boundaries between human-dominated and wild environments are increasingly blurred as human populations grow. The expanding interface between urbanised areas and the wild creates a situation where wild animals and humans live in close proximity. On the one hand, this interaction pushes many species to the limits of their natural habitats and threatens their survival. On the other hand, some species benefit and become urban invaders, thriving on the food and stability provided by human waste and infrastructure (1).

Urban invaders are potential sources of diseases. Wild animals living cryptically among humans provide new pathways for virus and pathogen transmission and their evolution (2). Emerging diseases in humans often result from pathogens jumping the animal-human boundary (3). This is a serious cause for concern; as the growing frequency of urban invaders raises the likelihood of wild animal-human contact, and hence the sharing or transmission of viruses and other pathogens.

The red (or European) fox (*Vulpes vulpes)* is an introduced species in Australia and has become a particularly adept urban invader. This generalist and highly adaptable species has spread throughout mainland Australia and has a significant impact on native species and ecosystems (4). Due to this adverse impact (4, 5) many ecological studies focus on rural or bushland populations of red foxes (6-10). The behaviour, ecology and potential health impact of Australia's urban foxes, however, has received little attention.

Wild red foxes are known to carry common canid diseases and parasites (11-14). However, in-depth analyses of the diversity of viruses and other pathogens that foxes might harbour are lacking. In Australia, urban foxes live in much higher densities than rural or bushland foxes (5, 15). Consequently, red foxes might go largely unnoticed in urban areas, yet be a significant reservoir of infectious agents. In this review I will discuss the evidence for, and impact of, disease transmission between wild animals and humans. I will review the literature on fox disease ecology with the aim of exploring the pathogen risk these urban invaders may pose to humans as well as other species.

Viruses, pathogens and the human-animal interface

Viruses and other pathogens exist in a dynamic world of constantly evolving hosts and changing environments. As hosts continually find ways to resist pathogen infection, pathogens find ways to overcome this (16). As a result, pathogen prevalence, virulence

and infective potential constantly change over time. This dynamic relationship between pathogens and their hosts sees the continuous emergence of novel diseases in humans and other animals. A global study conducted between 1940 and 2004 described over 300 emerging infectious disease events in humans. Of these emerging diseases, more than 60% originated in animals and at least 70% of these were from wild animals (3). Thus, wild animals play a particularly important role in disease emergence within human populations.

Some specific examples of well-known diseases that have had a significant impact on humans include: Ebola virus originating in bats; HIV/AIDS in non-human primates; the plague (black death) in rodents; and rabies virus in canines (17). These diseases have all jumped the animal-human barrier to infect human hosts. Disease outbreaks can have devastating effects on human populations, resulting in deaths and having other far-reaching consequences such as economic costs, impediments to trade and travel, and creating public fear. Although emerging disease events have been common throughout human history, and are a consequence of evolution and natural processes, the frequency of these events is increasing (3, 18)

A number of factors - social, biological and economic - influence the rate of emerging disease events in humans. For example, an aging population with people living longer increases the number of people that are vulnerable to infection. Increased social inequality can disproportionately affect the rate of emerging disease infection among disadvantaged groups (19). Increased global connectivity such as trade and travel create more routes and opportunities for pathogens to enter new areas and infect new hosts (20). The growing human population, encroachment on wild habitats and modification of natural landscapes, including urbanisation and agricultural production, likely influence disease emergence by raising the risk of contact between humans and animals, both wild and domestic (2, 21). The introduction of species to areas outside their native ranges and invasion of human dominated areas by opportunistic species also brings with it the risk of novel pathogen sharing and transmission (1, 22).

Despite the advancements of modern medicine, emerging diseases remain a constant threat to human health and wellbeing. Real-time genomic surveillance of pathogen transmission and disease emergence within human and animal populations is imperative for continued disease management within Australia and across the globe.

Urban ‘greening’

There is currently a global movement focused on ‘greening’ urban spaces. The initiative involves introducing more trees, plant life and parklands back into city regions. Green infrastructure in urban spaces or ‘nature-based solutions’ are frequently cited as ways to mitigate the impacts of climate change, promote sustainability, increase biodiversity and support human health and wellbeing (23, 24). Intuitively, greener cities mean more habitat which could potentially support a multitude of native and non-native species in novel ecosystems (25). Moreover, stable green spaces could provide urban invaders the opportunity to create permanent residences within urban spaces. In particular, larger mammals and carnivores are seizing the opportunity to take up residence in densely populated cities and live cryptically among humans (1). The consequences of such close contact with large, wild mammals, that are phylogenetically closer to humans than other urban invaders (e.g. birds, reptiles or invertebrates) remains largely unexplored.

The invasion of Australia by the red fox

The red fox, native to Europe and north America, is the most widely distributed carnivore on the planet. Its global range spans around 70 million km^2 and covers much of Eurasia, North America and Australia (26, 27). Although currently considered a single species, recent phylogenetic analysis has suggested that the North American population may be distinct (*Vulpes fulva)* due to extended reproductive isolation (26). With such a large global presence, the red fox could be considered one of the most adaptable and successful invasive species on Earth. Additionally, its broad range and ability to inhabit and thrive in multiple environments and habitats makes this species the perfect vector for the spread of disease.

Foxes were introduced to Australia by Europeans in the 1850s for sport hunting. After multiple introductions, the species quickly adapted to the Australian environment and became an established wild population in the 1870s (4). The red fox spread successfully throughout most of the Australian mainland, excluding only the most northerly regions (4). Current estimates puts the Australian red fox population at around 7.2 million animals (5). Foxes can survive in a wide range of habitats including rural and agricultural regions, natural and forested areas, arid and semi-arid interiors, as well as peri-urban and urban centres (4). Foxes are also known to inhabit major metropolitan regions, living in high densities in major city centres. In rural and bushland areas fox population densities only reach around 0.2 individuals per km^2 (5), whereas in Melbourne city foxes can reach

densities of up to 16 individuals per km^2 (15). A study in Bristol (UK) estimated that urban foxes can reach densities of up to 35 individuals per km^2 (28). Despite these high densities in Australia's urban areas, many people are unaware of their presence. Their cryptic and mostly nocturnal behaviour means they can operate within urban spaces while remaining largely unnoticed.

Foxes are mostly carnivorous with a highly generalist diet and are opportunistic hunters and scavengers. They consume a wide variety of food items including anthropogenic food sources and refuse, carrion, invertebrates, small mammals and birds, and occasionally fruit and vegetable matter (29-31). Foxes are surplus killers meaning they kill in excess of what is required for survival, caching food across multiple locations. Due to their predatory and caching behaviour, foxes have been linked to the declines and even extinctions of some Australian small mammal and bird species (4). Foxes have also been implicated in agricultural losses, including deaths of lambs and other juvenile livestock (4).

In Australia, urban fox ecology and behaviour have been scarcely explored compared to that of their rural counterparts. Indeed, much of the invasion ecology literature focuses on invasions of natural ecosystems, largely overlooking urban habitats. A database survey, looking at invasive species research up to the year 2015, revealed that less than 3% of published papers focused on urban areas (32). This lack of interest in urban invaders suggests that much is unknown about the ecology and impacts of invasive species that inhabit human dominated regions. The push to "green" cities and encourage native wildlife, including mammals, to return to urban areas may be negated by the presence of invasive species. The exclusion of native wildlife by foxes may be a hitherto unconsidered problem in urban habitats.

The role of foxes in pathogen transmission

With Australia's foxes living in such high densities in urban centres, a pertinent question is whether they pose a pathogen risk to humans as well as other domestic and native species (Figure 1). Foxes' ability to adapt to almost any environment suggests that they could be important carriers and transmitters of disease across multiple landscapes and ecosystems. Could foxes living in densely populated cities, with access to anthropogenic food sources and refuse, carry human associated pathogens that could be shared and spread across urban environments? Could pathogens that foxes pick up as a consequence of living in urban areas pose a risk to native wildlife along the urban-

bushland interface? Are foxes harbouring pathogens that are genetically close to human associated pathogens or have a history of jumping between different hosts? These are questions that warrant closer attention.

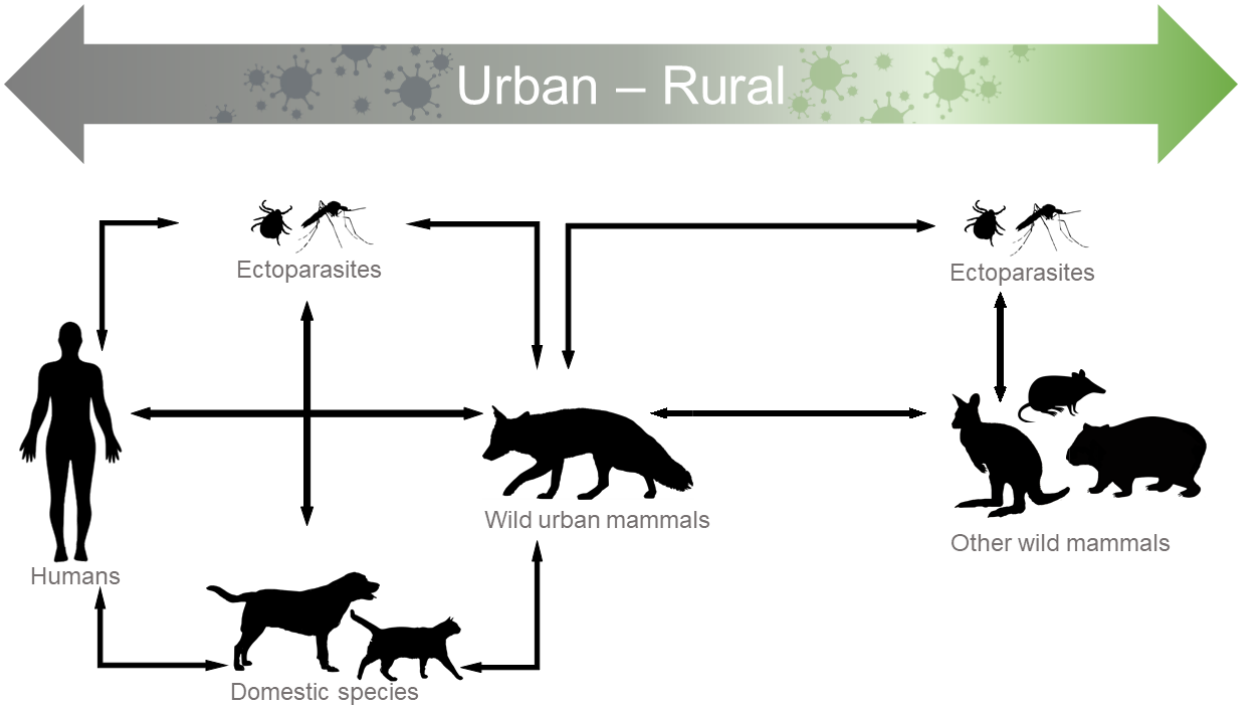

Figure 1. Potential pathways for pathogen transmission between urban wildlife, domestic species and humans.

Parasites and pathogens infecting foxes

(i) Bacteria

Across the European and North American continents, red foxes carry bacteria responsible for various infectious diseases in humans and other species. Some of these bacteria include: *Anaplasma phagocytophilum,* which can cause granulocytic ehrlichiosis in humans and other animals (33); *Bartonella* spp. which cause endocarditis in canines and can infect humans (34, 35); *Leptospira,* causing Weil's disease in humans (36); and *Mycobacterium bovis*, causing bovine tuberculosis in cattle as well as humans and other mammals (37). *Corynebacterium diphtheriae* which causes diphtheria, usually exclusively in humans, and the Lyme disease causing bacterium, *Borrelia burgdorferi*, have also been detected in wild foxes in Germany (38, 39).

(ii) Helminths

Parasitic helminths such as nematodes, cestodes and trematodes are common in foxes around the globe (40-46). The parasitic tape-worm *Echinococcus multilocularis,* known to cause serious infection in humans and other mammals, is common in foxes in the northern hemisphere (47-52). This particular tapeworm is also common in foxes living in urban regions (53, 54). *Echinococcus granulosus*, a similar parasite, has been found in foxes in Australia's Mt Kosciusko national park and around the Canberra region (55, 56). Heartworm, a parasitic worm infection affecting domestic dogs, has also been found in Australian foxes near the Sydney area and in Melbourne city (57, 58). Trichinella is a parasitic nematode that causes severe muscular infection in humans and other mammals, and is found commonly in foxes across Europe (14, 59-61).

(iii) Protozoan and other microscopic parasites

Various protozoan and other microscopic parasites infect foxes. *Toxoplasma gondIi*, which has been linked to behavioural and neurological disorders in humans, has been found in foxes based on the presence of antibodies (13, 62). *Neospora canium,* a parasite implicated in the cause of spontaneous cattle abortion and posing a serious problem for agricultural production, is suspected to inhabit foxes - however, evidence for this is limited. Serological studies looking for antibodies to *N. canium* have found some evidence of an extremely low prevalence in European foxes (13, 62). Analysis of brain tissue and faecal samples from foxes in Spain found evidence for *N. canium* in brain tissue by PCR, however, no oocysts were detected in faecal samples (63). An experimental study that purposely infected foxes with *N. canium* came to the conclusion that foxes were either inappropriate hosts, or not definitive hosts for this parasite (64). Other notable fox protozoan and microscopic pathogens that can cause infection and illness in humans include *Babesia* spp., *Theileria* spp., *Giardia, Cryptosporidium* and *Leishmania* spp. (61, 65-68).

(iv) Viruses

Rabies virus is one of the most well-known canine viruses that can cause serious problems for the health and wellbeing of humans, domestic dogs and other wild canines. Though eradicated in some regions, rabies virus has been documented in foxes across Europe and North America (69). If rabies virus ever made its way to Australia, foxes represent a viable vector for the disease to spread across the continent. The only viruses documented in Australian foxes so far are: Trubanaman virus, causing polyarthritis-like symptoms in infected humans (70); canine herpesvirus, affecting mostly domestic dogs;

and canine adenovirus, affecting mostly mammals (71). Other canine viral pathogens found in foxes in Europe include canine distemper virus and canine parvovirus (72, 73). A number of novel viruses in red foxes have been discovered in the Netherlands and Croatia, including adeno-associated virus, bocavirus, parvoviruses, hepevirus, astroviruses, picobirnaviruses and circoviruses, some of which are close relatives of human-associated viruses (73, 74). This finding suggests that foxes and humans may have transmitted viruses between one another in the past (73, 74). As mammals, foxes and humans are phylogenetically close and viruses may jump more easily between similar hosts (75). In urban areas where foxes live among humans, there is increasing opportunity for novel viral transmission.

In 2008, a research group in the Netherlands experimentally infected foxes with avian influenza virus (H5N1) by feeding them infected bird carcasses. They found foxes could excrete the virus for up to 5 days after infection with little or no symptoms (76). This finding is particularly concerning as it suggests foxes can serve as hosts for the highly pathogenic influenza virus and act as viral dispersers without any serious health impediment.

The limited number of viral studies in foxes, particularly in urban areas, makes it hard to know the true viral risk that these cryptic urban invaders might pose to humans.

(v) Ectoparasites

Foxes are host to numerous ectoparasites including fleas, ticks and mites (12, 39). Foxes can therefore assist in the transmission of vector-borne diseases by providing stable environments for these parasites and spreading them throughout urban areas. Sarcoptic mange is common in foxes and can be spread to domestic and wild mammals (12, 39, 77, 78). The disease can be deadly and has had a significant impact on the wombat population in Australia (79). Recently, sarcoptic mange has also been identified in other Australian marsupials, including koalas (*Phascolarctus cinereus*), and is suspected to result from a ‘spill-over’ event involving foxes and domestic and wild dogs as reservoirs (80). A ‘spill-over’ event refers to the transmission of a pathogen from its original host species to another. The recent emergence of sarcoptic mange in Australia’s marsupials’ points to the potential for serious infectious diseases to jump the barrier between wild animal species and pose a significant threat to endangered species.

Methods for detecting fox pathogens

To date, the main methods used to carry out pathogen studies in red foxes have included: direct observation of the pathogen at particular life stages; DNA identification by PCR; serological analysis of antibodies; and observation of disease pathology (e.g. lesions or other tissue damage). Many studies have focused on finding a particular disease-causing pathogen or a particular parasite. With the increased refinement and accessibility of modern technologies such as next generation sequencing, there are greater opportunities to better describe the fox 'infectome'. Urban foxes are underrepresented in pathogen research. Implementing these technologies, particularly in urban foxes, will be advantageous for monitoring emerging diseases in urban environments.

Meta-transcriptomics, for example, is a next-generation sequencing method which can reveal the entire transcriptome of an animal - this includes identifying the viruses and other pathogens present as well as their abundance (81-83). Using meta-transcriptomics to completely (i.e. without bias) describe the fox 'infectome' could assist in identifying novel pathogens and understanding trends in emerging infectious diseases. To our knowledge, this particular method has not yet been applied to red foxes.

Conclusion

Thus far, red fox disease research in Europe and North America has focused mainly on identifying specific pathogens or classes of pathogens; the most well studied system being parasitic helminths. Research on the other pathogens (including viruses) that foxes might carry does exist, however it is limited in scope. Broadening the research horizon on pathogen research in red foxes, including the implementation of novel techniques such as meta-transcriptomics, will enhance our ability to effectively assess and monitor foxes as a potential reservoir of emerging pathogens.

As the urban-wild interface expands, contact between humans and wild animals becomes increasingly likely. The dynamic nature of disease emergence, and the serious threat it poses to human health, makes understanding and monitoring pathogens and diseases in urban wildlife extremely important. It is clear that virus and pathogen research in Australia's red foxes is lacking. In terms of Australia's urban foxes, virus research is essentially non-existent. As fox populations continue to grow in urban areas there is a significant possibility that these animals are becoming important vectors and spreaders of disease.

Foxes are living on our doorsteps. Exploring the pathogens they carry may prove extremely important for future disease monitoring and management.

Chapter II: A comparison of red fox viromes across an urban-rural gradient

This chapter is written as a manuscript for submission to *Journal of Virology* and is formatted accordingly. Figures have been inserted within the text at appropriate positions to allow ease of reading and comprehension. Line numbers within the manuscript have been removed to conform with the rest of the thesis.

A comparison of red fox viromes across an urban-rural gradient

Sarah J Campbell[a,#], Wilbur Ashley[a], Thomas Newsome[b], Margarita Gill-Fernandez[a], Francesca Di Giallonardo[c], Jackie Mahar[d], Michael Gillings[a], Alexandra Carthey[a], Jemma L Geoghegan[a]

[a]Department of Biological Sciences, Macquarie University, Sydney, NSW 2109, Australia.

[b]School of Life and Environmental Sciences, The University of Sydney, Sydney, NSW 2006, Australia.

[c]The Kirby Institute, University of New South Wales, Sydney, NSW 2052, Australia.

[d]Marie Bashir Institute for Infectious Diseases and Biosecurity, Charles Perkins Centre, School of Life and Environmental Sciences and Sydney Medical School, The University of Sydney, Sydney, NSW 2006, Australia.

[#]sarah.campbell4@hdr.mq.edu.au

Running title: The red fox virome

Abstract

Red foxes have established abundant populations in Australia's urban and rural areas since their introduction following European settlement. Their cryptic and highly adaptable nature means they can invade city centres and live among humans while remaining largely unnoticed. Urban living and access to anthropogenic resources can influence fox ecology. Urban foxes grow larger, live in higher densities and are more social than their rural counterparts. These ecological changes in urban red foxes are likely to impact the pathogens that they harbour and may mean that foxes pose a disease risk to humans and other species that share these urban spaces. To assess this possibility, we characterised the virome of urban and rural foxes across the Greater Sydney region, Australia, using a meta-transcriptomics approach. We found that urban and rural foxes differed significantly in virome composition and that rural foxes harboured a greater abundance of viruses compared to urban foxes. We identified potentially novel vertebrate-associated viruses in both urban and rural foxes, some of which are close relatives of viruses that are associated with disease in domestic species and humans. This study has shed light on the pathogens that urban and rural foxes harbour and emphasises the need for greater genomic surveillance of foxes and other invasive species at the human-wildlife interface.

Importance

Urbanisation of wild environments is increasing as human populations continue to expand. Remnant pockets of natural environments and other green spaces in urban landscapes provide invasive wildlife with refuges where they can colonise urban areas and thrive on the resources provisioned by humans. Close contact between humans, domestic species and red foxes likely increases the risk of novel pathogen emergence. Indeed, the vast majority of emerging infectious diseases in humans originate in wild animals. Pathogen surveillance in urban wildlife is vital for monitoring viral disease and for the prevention of viral emergence.

Key words: *Vulpes vulpes*, carnivore, exotic species, urban adapter

Introduction

Red foxes (*Vulpes vulpes*) have the largest natural distribution of all wild terrestrial carnivores (27). Their native range extends throughout Eurasia and north America and an introduced population exists in Australia (26). Introduced in the mid-1800's, red foxes have spread throughout most of the Australian continent. They are one of the most highly adaptable species on the planet and can exploit a wide range of climates and habitats, from alpine to desert. Foxes are also broadly distributed across different land uses including natural and forested landscapes as well as highly urbanised, human dominated landscapes (1, 4).

Fox home range size varies depending on habitat and land use. Globally, average urban fox home ranges are approximately 1.7 km^2, while rural fox home ranges average around 5.7 km^2. Rural home ranges as large as 19.9km^2 have been recorded in north America (84). In Australia, home ranges for foxes in arid regions can reach up to 20km^2 (4), between 5-7km^2 in rural areas (85) and less than 1km^2 in urban centres (86). Foxes are common across rural and bushland regions in Australia and have established a large presence in major metropolitan centres (4, 15). Foxes have been recorded near the Sydney region since 1907 (87). They were first sighted in an Australian city (Melbourne) in 1943, although they were sighted in Melbourne's suburban surrounds as early as 1933 (88). For comparison, foxes were first noted in British cities (i.e., in their native range) in 1930 (1).

Fox control

Predation of native animals by red foxes has been identified as a key threat to Australian native ecosystems (89). The list of native animals threatened by fox predation include some of Australia's most endangered species, such as the brush-tailed bettong (*Bettongia penicillate*), rufous hare-wallaby (*Lagorchestes hirsutus*) and loggerhead turtle (*Caretta caretta*), as well as the critically endangered Gilbert's potoroo (*Potorous gilbertii*), western swamp tortoise (*Pseudemydura umbrina*) and orange bellied parrot (*Neophema chrysogaster*) (90). Due to the threat foxes pose to endangered wildlife and Australian biodiversity, fox populations are actively controlled.

Control methods for foxes depend on habitat and land use. Poison baiting is the most common and cost-effective method of eradication in rural areas (4). In urban areas however, the risk to pets limits control methods to trapping and shooting (91). Foxes are notoriously difficult to trap, and shooting in urban areas requires tracking at night (when foxes are most active) by licensed professionals. Such limitations make effective fox control in urban areas difficult.

Foxes likely inhabit natural landscapes scattered throughout cities and their highly adaptable nature means they can also exploit the rich anthropogenic food resources in their urban surrounds. Red foxes are mostly nocturnal, residing in dens during the day and emerging at night to hunt and scavenge (92, 93). In an urban setting, foxes remain diurnally inactive, only venturing out at night. This cryptic behaviour means they are scarcely noticed in urban areas despite their high abundance. Due to the perceived minimal activity of foxes in Australian cities there is limited research exploring the effects of fox presence in urban areas (but see (15, 86, 88, 94, 95)).

Urban foxes

Foxes thrive on the resources inadvertently provided by humans in cities (1, 31, 94). Consequently, foxes and other carnivores in urban areas with access to these resources can experience different selection pressures compared to their rural counterparts. This can result in distinct urban behaviours such as increased boldness and decreased human aversion (1). These changes in behaviour may make human encounters more likely. Urban living also increases carnivore body size which may have positive effects on fitness and fecundity (1, 95). Indeed, foxes in urban areas in the Greater Sydney region are significantly larger and heavier than foxes in rural areas (95).

When food is abundant, carnivore home ranges are smaller, higher densities are supported and encounters between conspecifics are more frequent (1, 96). In urban areas, fox family groups are often larger than those in rural areas with juvenile females remaining in their natal territory to assist with rearing subsequent litters (15, 97, 98). Thus urban environments may favour increased conspecific tolerance and social behaviours in foxes which are usually more solitary in rural areas (15, 97, 98).

Viral risk

Red foxes harbour a diversity of viruses (73, 74). However, potential differences in viromes between urban and rural foxes remains largely unexplored. High-density living and increased host contact can increase pathogen transmission rates among hosts (99) . Therefore, a high-density population of cryptic urban foxes living in close proximity to largely unsuspecting humans could pose an important pathogen risk. Foxes are likely to investigate human refuse and consume scraps from surfaces such as outdoor barbeques and furniture, eat from dog bowls and defecate nearby, increasing the potential for pathogen transfer (31). In addition, as urban animals can become habituated to humans with passing time (1), we would expect to see an increase in direct fox-human interactions, and hence for the potential transmission of disease between the two species.

Novel viruses pose a significant health threat to humans as well as domestic species and native wildlife (100). Several novel viruses infect humans each year and viruses account for two-thirds of all newly discovered human pathogens (101). Since most human infectious diseases tend to originate in wildlife (3), viral surveillance of urban species may be particularly important in monitoring and preventing disease emergence and spread.

Meta-transcriptomics for viral discovery in red foxes

Meta-transcriptomics is a next generation sequencing technology that has been successfully used to describe the virome of both vertebrate and invertebrate hosts (81, 102-105). This approach involves deep sequencing of the host transcriptome and enables identification of the viruses present and their relative abundances. Using this approach, we describe the Sydney red fox virome and compare the viromes of urban and rural foxes. Due to the presumed higher population densities and increased conspecific interactions in urban areas, we hypothesise that foxes in urban areas could harbour a greater viral diversity and abundance compared to rural foxes. In addition, due to female fox social behaviour such as cooperative cub rearing (97, 98), we postulate that females could harbour a greater diversity and abundance of viruses than more solitary males.

To this end, samples (liver, faecal and ectoparasite) were collected from foxes around the Greater Sydney region (Figure 1). Samples were pooled (based on sampling location and sex) and subject to RNA sequencing to reveal viral diversity, evolution and abundance.

This is the first time meta-transcriptomics has been used to describe the virome of the introduced Australian red fox.

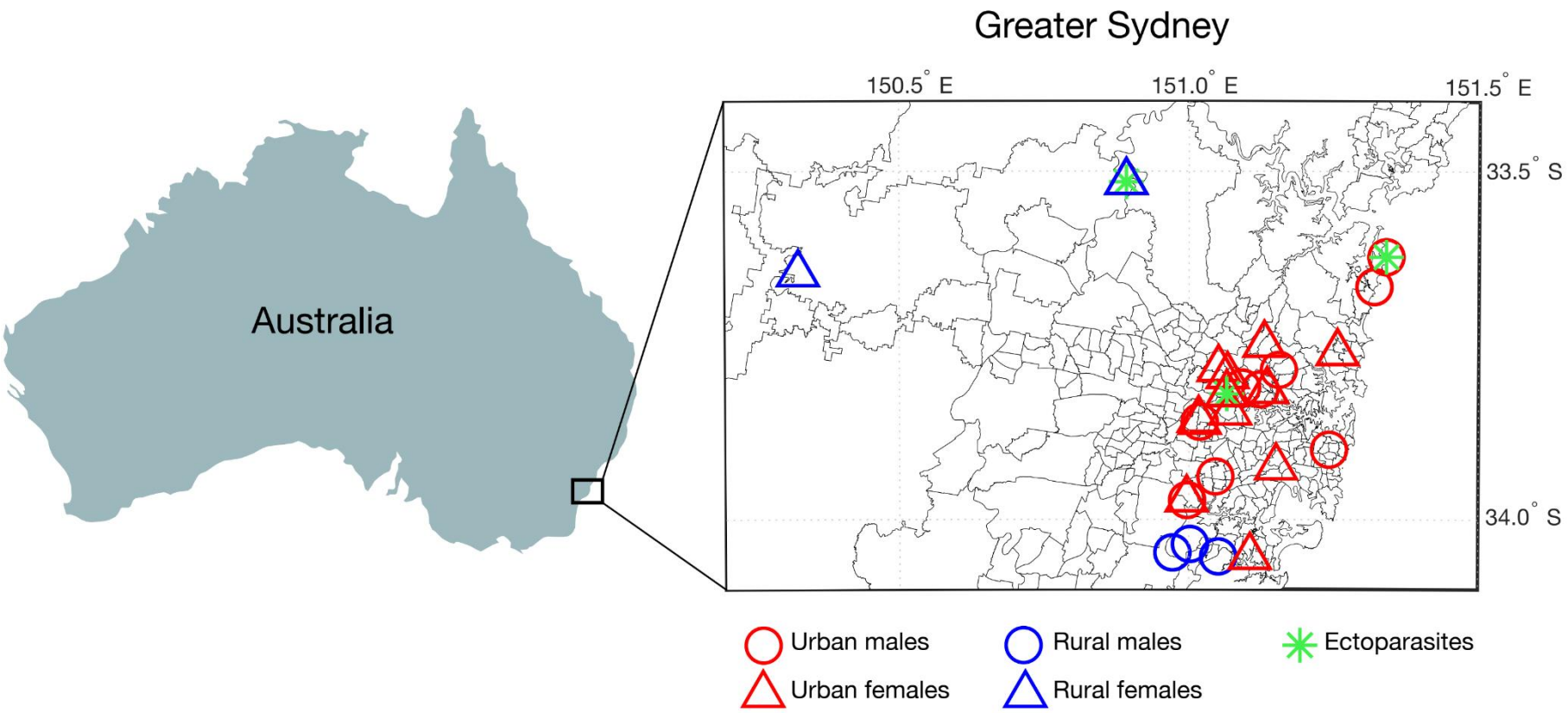

Figure 1. Map of the Greater Sydney region showing fox sampling locations of urban (red) and rural (blue) fox carcasses, identified as male (circle) or female (triangle), as well as those harbouring ectoparasites (green).

Materials & Methods

Sample collection

The current project was part of a larger research program into urban foxes in partnership with Greater Sydney Local Land Services. As such, we arranged to collect fresh fox carcasses killed by pest controllers in the Greater Sydney region (Figure 1). Each fox was collected as soon as possible after death (03:19:00 ± 02:59:00 hrs after death, n=27) to minimise degradation of RNA in samples. One carcass had been frozen for approximately one week and one carcass had been dead for an unknown amount of time. The foxes used for this study were either trapped in a cage and shot, or tracked and shot. One individual was obtained as recent roadkill. Foxes killed by poison baits were not included in this study.

Whole fox carcasses were collected and transported to the laboratory where they were dissected to collect faecal, liver and ectoparasite samples. All samples were stored separately in RNALater and at -80°C. We sampled a total of 29 individual foxes; 13 males and 16 females. The average body mass of an adult red fox is approximately 5.5kg and can range between 3.3 and 8.2kg. Average body length is approximately 64cm and can range between 51 and 78cm (when measured from the tip of the nose to the first vertebra of the tail) (106). For this study, foxes were classified as juvenile if their body mass and body length were less than 3.3 kg and 51cm, respectively. Based on this assessment, 25 foxes were classified as adults (12 males, 13 females) and four as juveniles (1 male, 3 females).

Sampling in urban and rural areas

Fox sampling relied largely on coordination with professional pest control operators who focus control efforts in specific locations in accordance with local control initiatives. For this reason, a comprehensive land-use gradient from urban to rural could not be fully achieved. Sufficiently fresh rural and bushland fox samples were also difficult to obtain since poison baiting is the principal control method in these areas. Therefore, ‘rural’ was broadly defined as any natural bushland, national park, mostly agricultural or sparsely populated region outside the central urban districts, with a human population density of fewer than 500 people per km^2. ‘Urban’ was defined as built up areas inside the central urban district

(including parks, gardens and golf courses) with a population density of more than 500 people per km^2 either in the area sampled or in the immediate surrounding areas. Human population density information was obtained from the Australian Bureau of Statistics (2016 census data) (107). Central urban districts were defined by the Urban Centres and Localities statistical classification (UCL) (108). Land use classification and human population density cut-offs were loosely based on work by Stepkovitch (2019).

RNA extraction

Qiagen RNeasy Plus Mini Kits were used to extract RNA from liver, faecal, and ectoparasite samples from collected red foxes. Thawed samples were transferred to a lysis buffer solution containing 1% β-mercaptoethanol and 0.5% Reagent DX. Samples were homogenized and centrifuged. DNA was removed from the supernatant via gDNA eliminator spin column and RNA was eluted via RNeasy spin column. RNA concentration and purity were measured using the Thermo Fisher Nanodrop.

Library preparation and whole transcriptome sequencing

Samples were pooled based on land use category (urban or rural), sex and sample type (liver, faecal or ectoparasite), resulting in nine representative sample pools (Table 1). Adults and juveniles were pooled together since so few juveniles were sampled.

The TruSeq Stranded Total RNA Ribo-Zero Gold (h/m/r) kit was used to prepare pooled samples for sequencing. Pooled samples were sequenced on the NextSeq 500 with 2x75bp output at the Ramaciotti Centre for Genomics at the University of New South Wales, Sydney. Sequencing resulted in nine representative data libraries (Table 1), which will be available on NCBI's SRA database upon publication.

Table 1. Breakdown of red fox representative samples, detailing land-use, sex and sample type, as well as the number of individuals pooled for RNA sequencing.

Representative sample	Land use	Sex	Sample type	Number of individual foxes pooled
1	urban	male	liver	9
2	urban	male	faeces	6
3	rural	male	liver	3
4	rural	male	faeces	3
5	urban	female	liver	9
6	urban	female	faeces	13
7	rural	female	liver	3
8	rural	female	faeces	3
9	both	male (1) female (2)	ectoparasites	3

Virus discovery

Short sequencing reads were assembled *de novo* into longer sequences (contigs) based on overlapping nucleotide regions using Trinity RNA-Seq (109). Assembled contigs were assigned to a taxonomic group (virus, bacteria, archaea, eukarya) and viruses were identified to their closest species match based on sequence similarity searches against the

NCBI nucleotide (nt) and non-redundant protein (nr) databases using BLASTn (110) and Diamond (BLASTX) (111), respectively. Non-viral hits such as host contigs with similarity to viral sequences (e.g. endogenous viral elements in the fox genome) were removed.

Inferring the evolutionary history of fox viruses

The phylogenetic relationships of the vertebrate-associated viruses identified in the samples were estimated. First, the amino acid translations of the viral transcripts were combined with other protein sequences obtained from GenBank (Table 2). Second, the sequences were aligned using MAFFT v.3.4, employing the E-INS-I algorithm. Ambiguously aligned regions were removed using trimAl v.1.2 (112). To estimate phylogenetic trees, we selected the optimal model of amino acid substitution identified using the Bayesian Information Criterion as implemented in Modelgenerator v0.85 (113) and employed the maximum likelihood approach available in PhyML v3.1 (114) with 1000 bootstrap replicates.

Diversity and abundance analysis

Transcript abundance was estimated using RSEM within Trinity (115). This approach assessed how many short reads within a given library mapped to a particular transcript. Raw counts were then standardised against the total number of reads within each library. Virome diversity and relative abundance were compared among samples using a non-metric multidimensional scaling (nMDS) ordination in conjunction with an analysis of similarities (ANOSIM) using the vegan package in R (116). To determine which viral families were contributing the most to differences between samples, an indicator species analysis was performed using the indicspecies package in R (117).

Results

Overview

Meta-transcriptomic sequencing of nine representative pooled samples resulted in 44-57 million paired reads per pool (593,406,706 reads in total). The BLAST analyses revealed

that all faecal samples were dominated by bacteria (51.17-84.61%), while the liver samples were dominated by eukarya (92.90-99.43%), comprising mostly fox RNA. Viruses made up a small proportion of the four representative faecal samples (0.002-5.85%) and were detected in only one of the representative liver samples (0.001%). Archaea were detected at very low levels in faecal samples only (0.002-0.021%). The ectoparasites differed substantially to the liver and faecal samples with 50.97% of reads classed as 'unmatched' meaning they did not share sequence similarity to any known sequence. The remainder of the ectoparasite samples consisted of eukarya (44.39%), bacteria (4.64%) and viruses (0.004%). Unmatched reads in liver and faecal samples ranged between 0.52-12.22% (Figure 2a).

Multiple novel vertebrate-associated virus transcripts were identified from both urban and rural foxes, including a hepevirus, picobirnavirus, astrovirus, rabbit haemorrhagic disease virus-2 (RHDV2) and various picornaviruses (Table 2). Vertebrate-associated virus transcripts represented between 0.4-98% of viral reads in faecal samples. The remainder comprised mostly invertebrate, plant and fungi associated virus transcripts which were most likely acquired from the foxes' diet.

Virome composition

The urban, rural and ectoparasite samples were clearly distinct (Figure 3). A dissimilarity matrix showed that there was a clear separation between the three sample types suggesting that their viral composition differed significantly ($p<0.05$). The viral families responsible for these differences were the *Picornaviridae* in the rural samples and the *Nodaviridae* in the urban samples. It must be noted that fewer data points increase the chances of a significant result, however, this is balanced by the fact that the representative samples each contain multiple individuals.

Urban and rural fox viromes

Transcripts from a total of 21 distinct viral families were identified in urban foxes and 19 in rural foxes. Urban fox samples showed a more even diversity of viruses compared to rural fox samples, which were heavily dominated by the *Picornaviridae*. Picornaviruses (including picorna-like viruses) made up between 77.33-98.97% of rural fox samples

(Figure 2b). On average, total viral abundance was higher in rural samples (2.03 ± 3.31%, n=3) than urban samples (0.03 ± 0.04%, n=2) (Figure 2c).

While vertebrate-associated viruses were found in both urban and rural faecal samples, an average of 98.69 ± 1.15% (n=2) of viruses in the rural samples were vertebrate-associated, compared to just 1.4 ± 1.43% (n=2) in urban samples. The majority of reads from urban fox samples were invertebrate, plant or fungi associated viruses.

Male and female fox viromes

On average, total viral abundance was higher in female foxes (1.97 ± 3.36%, n=3) than in male foxes (0.12 ± 0.17%, n=2) (Figure 2c). Viral diversity was also higher in females (25 distinct viral families) than in males (13 distinct viral families). While vertebrate-associated viruses were found in both male and female faecal samples, the percentage of viruses that were vertebrate-associated were far higher in rural foxes (male: 98.23%, female: 97.84%) than in urban foxes (male: 2.41%, female: 0.39%).

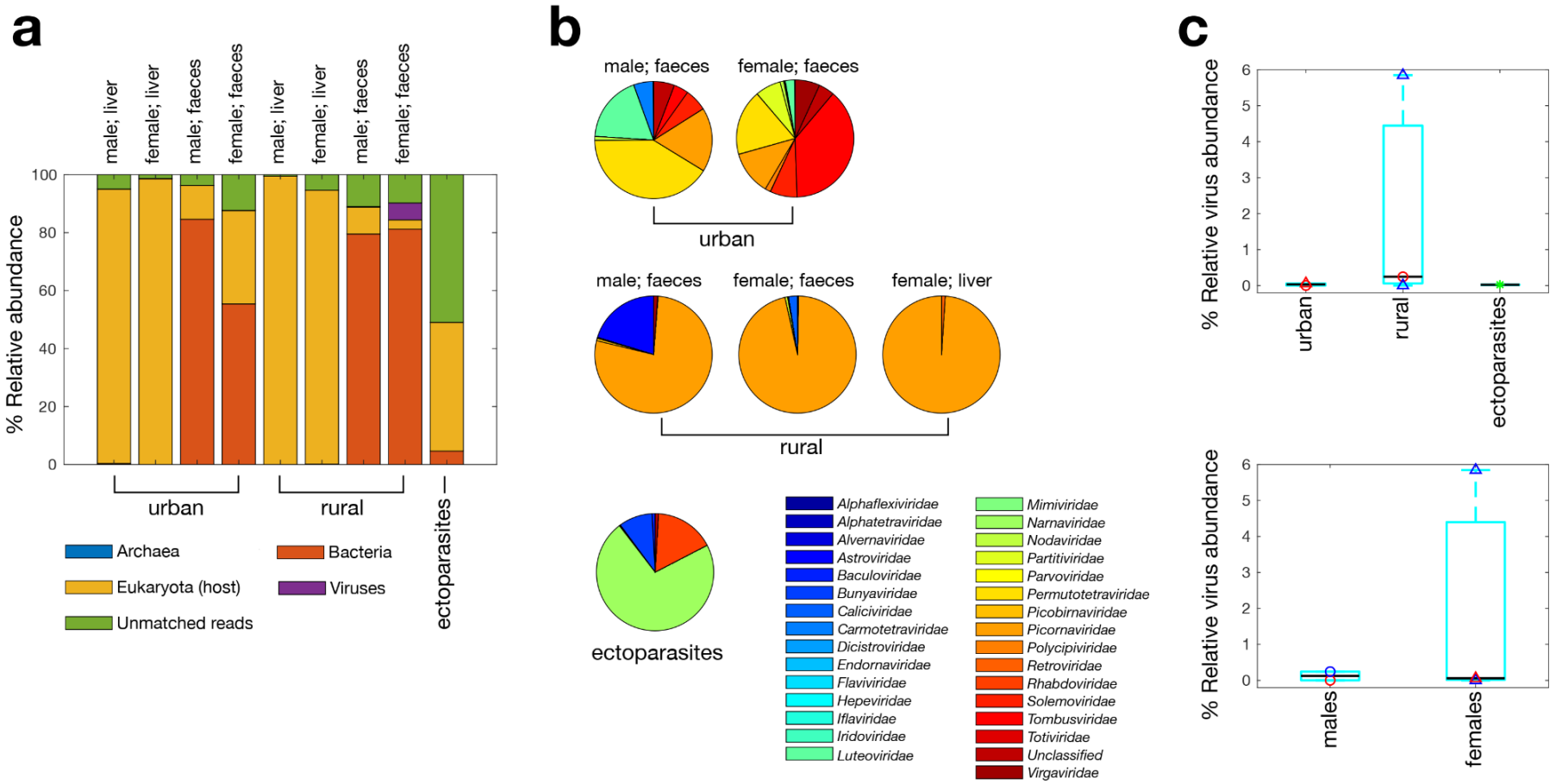

Figure 2. An overview of the red fox virome. (a) Percentage abundance of each taxonomic group identified in each respective pooled sample, standardised against the number of raw reads per pool. Due to their low abundance, archaea (0.002-0.021%) and some of the viral reads (0.001-5.85%) are too small to see. (b) Percentage abundance of (eukaryotic-associated) viral families detected in each respective pooled sample (excluding bacteriophage). (c) Boxplots showing percentage abundance of (eukaryotic-associated) viral reads in urban, rural and ectoparasite samples and males and females. A black line indicates the median and the bottom and top edges of the box indicate the 25th and 75th percentiles, respectively. Raw abundances are superimposed, and the colour and shape of data points are as in Figure 1.

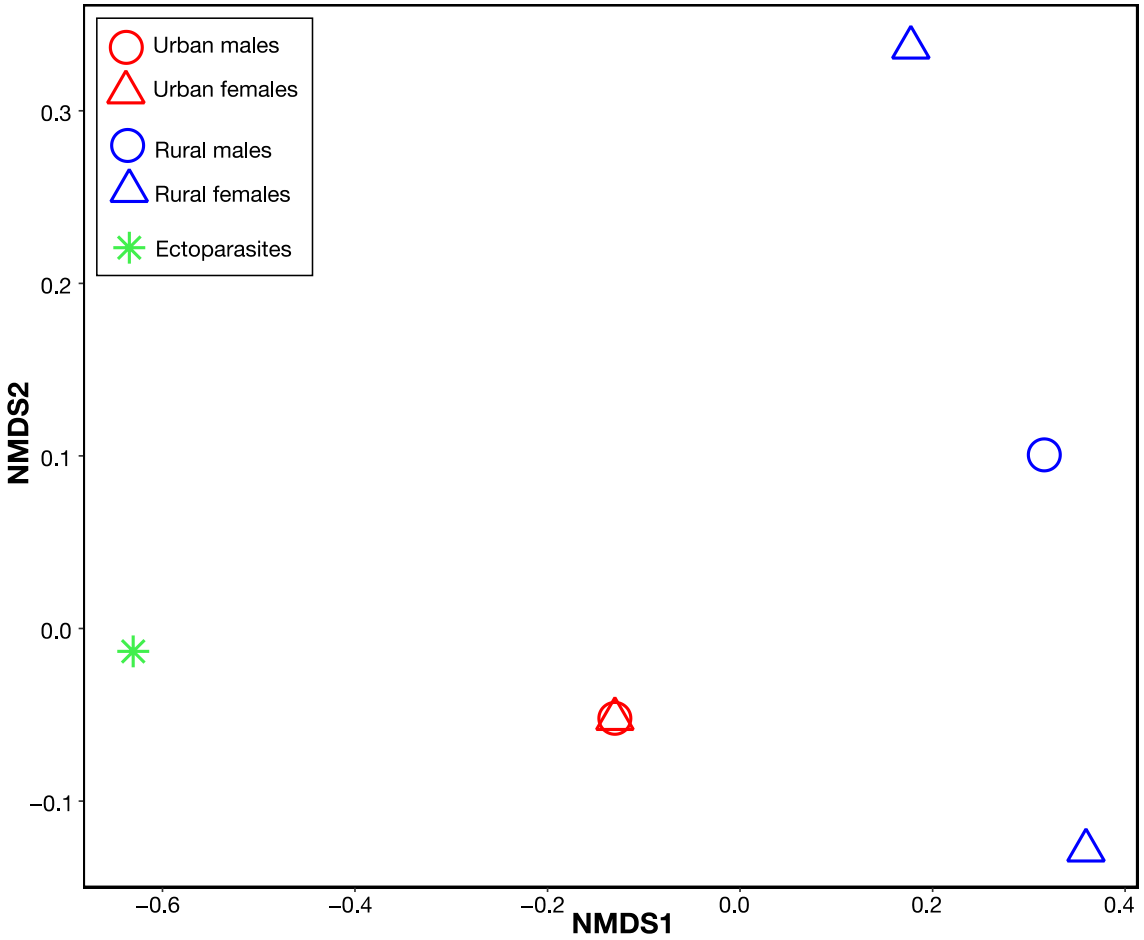

Figure 3. Non-metric multidimensional scaling (nMDS) ordination showing differences in virome composition (at the family level) among samples according to habitat and sex. Individual points represent individual pooled samples. Points closer together have a more similar virome composition (based on diversity and abundance of viruses) and *vice versa* for those further apart.

Vertebrate-associated viruses

(i) Hepeviridae

Hepevirus sequences were discovered in the rural female faecal samples. Tentatively named red fox hepevirus, this virus transcript shared 28.92% amino acid identity to elicom virus-1 in mussels (Table 2). Phylogenetic analysis of the RNA dependant RNA polymerase (RdRp) encoding region placed this hepevirus in close proximity to both house mouse hepevirus and elicom virus-1 (Figure 4).

(ii) Astroviridae

We detected an astrovirus, tentatively named red fox astrovirus-2, in the rural male faecal samples. The sequence shared a 96.11% amino acid identity with feline astrovirus D1 (Table 2). Based on phylogenetic analysis of the RdRp, red fox astrovirus-2 clustered with other mammalian associated mamastroviruses (Figure 4).

(iii) Picobirnaviridae

Picobirnavirus related sequences were detected in urban male, rural male and urban female faecal samples. As some of the sequences were short, or represented less conserved regions of the viral gene, only one sequence (from the urban female samples) was used for phylogenetic analysis. The sequence, tentatively named red fox picorbirnavirus-2, shared an 80.27% amino acid identity with a picobirnavirus found in wolves (Table 2). The sequence also clustered with other mammalian associated picobirnaviruses (Figure 4).

(iv) Picornaviridae

Two kobuvirus related sequences were discovered in the rural female faecal samples. The longer sequence, tentatively named fox kobuvirus-2, shared the highest amino acid identity with canine kobuvirus from a domestic dog (97.65%) (Table 2). Analysis of the RdRp region showed the sequence clustered most closely with feline kobuvirus and other mammalian kobuviruses (Figure 4).

Multiple picodicistrovirus related sequences were detected in the urban male, rural male and urban female faecal samples. Three of the sequences (two from the rural male and one from the urban female samples), tentatively named red fox picodicistrovirus-1 ,2 and 3, all shared more than 94% amino acid identity with canine picodicistrovirus (Table 2).

Based on analysis of the RdRp region all three sequences clustered together with mammalian dicipivirus and rosaviruses as well as reptilian picornaviruses (Figure 4).

Multiple picornavirus related sequences were identified in the urban male and rural female faecal samples as well as the rural female liver samples. Three sequences from each representative sample, tentatively named red fox picornavirus-1, 2 and 3, all shared between 73.37-96.98% amino acid identity with canine picornavirus (Table 2). All three sequences clustered with other mammalian picornaviruses in the order *Sapelovirus* (Figure 4).

(v) Caliciviridae

Rabbit haemorrhagic disease virus (RHDV) was detected in rural female and urban male faecal samples. The viral sequence in the rural female samples shared a 99.62% amino acid identity with RHDV2 isolated from rabbits between 2015-2016 (Table 2) (Figure 5). The viral sequence in the urban male samples was too short to enable phylogenetic analysis. This is the second time that RHDV has been found in non-rabbit hosts (102), presumably in this case through rabbit consumption.

Table 2. Vertebrate-associated viral contigs, contig length (nt), percent abundance in their respective pools and the percent amino acid identity to their closest match on GenBank.

Host	Land use (sex)	Virus species	Contig length (nt)	% Relative abundance	Closest match (GenBank accession number)	% Amino acid identity
Red fox (*Vulpes vulpes*)	rural (female)	Red fox kobuvirus	2427	0.007%	Canine kobuvirus (AZS64124.1)	97.65%
		Red fox picornavirus-1	1428	0.0003%	Canine picornavirus (YP_005352651.)	96.98%
		Red fox picornavirus-2	7236	5.66%	Canine picornavirus (YP_005351240.)	89.18%
		Red fox hepevirus	7374	0.01%	Elicom virus-1 (YP_009553584.)	28.92%
		Red fox associated rabbit haemorrhagic disease virus-2	7026	0.14%	Rabbit haemorrhagic disease virus (MF421679.1)	99.62%
	rural (male)	Red fox picodicistrovirus -2	4263	0.17%	Canine picodicistrovirus (YP_007947664.)	98.53%
		Red fox picodicistrovirus -3	300	0.0003%	Canine picodicistrovirus (AFB77699.1)	95.92%
		Red fox astrovirus-2	2556	0.046%	Feline astrovirus (YP_009052460.)	96.11%
	urban (female)	Red fox picodicistrovirus -1	2062	0.0004%	Canine picodicistrovirus (YP_007947664.)	98.83%
		Red fox picobirnavirus-2	448	0.0001%	Wolf picobirnavirus (ANS53886.1)	80.27%
	urban (male)	Red fox picornavirus-3	1524	0.00058%	Canine picornavirus (YP_005351240.)	73.37%

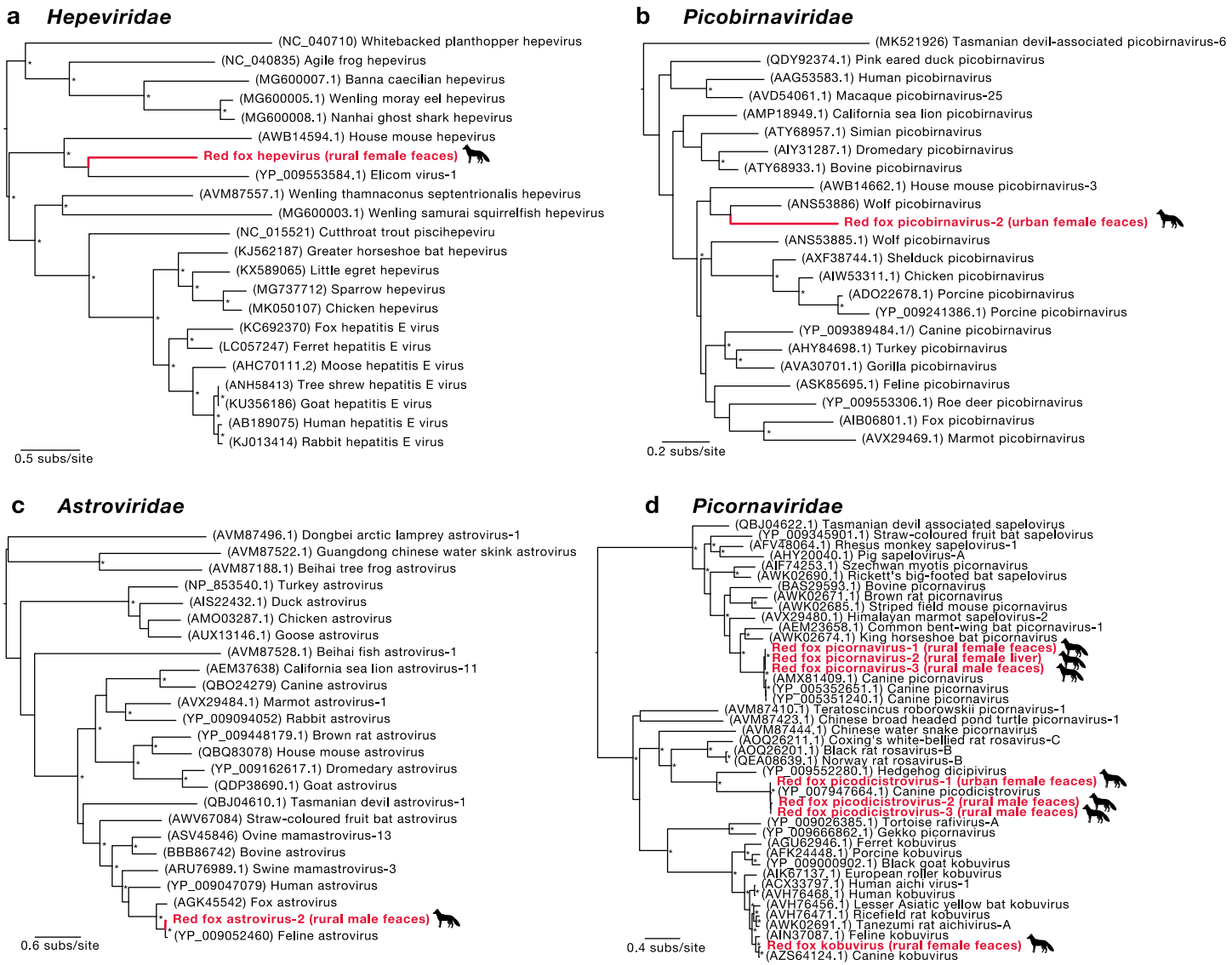

Figure 4. Phylogenetic relationships of likely vertebrate-associated viruses discovered from assembled contigs: (a) *Hepeviridae*, (b) *Picobirnaviridae*, (c) *Astroviridae* and (d) *Picornaviridae*. The maximum likelihood phylogenetic trees show the topological position of the newly discovered potential viruses (bold, red text), in the context of their closest relatives. All branches are scaled to the number of amino acid substitutions per site and trees were mid-point rooted for clarity only. An asterisk indicates node support of >70% bootstrap support.

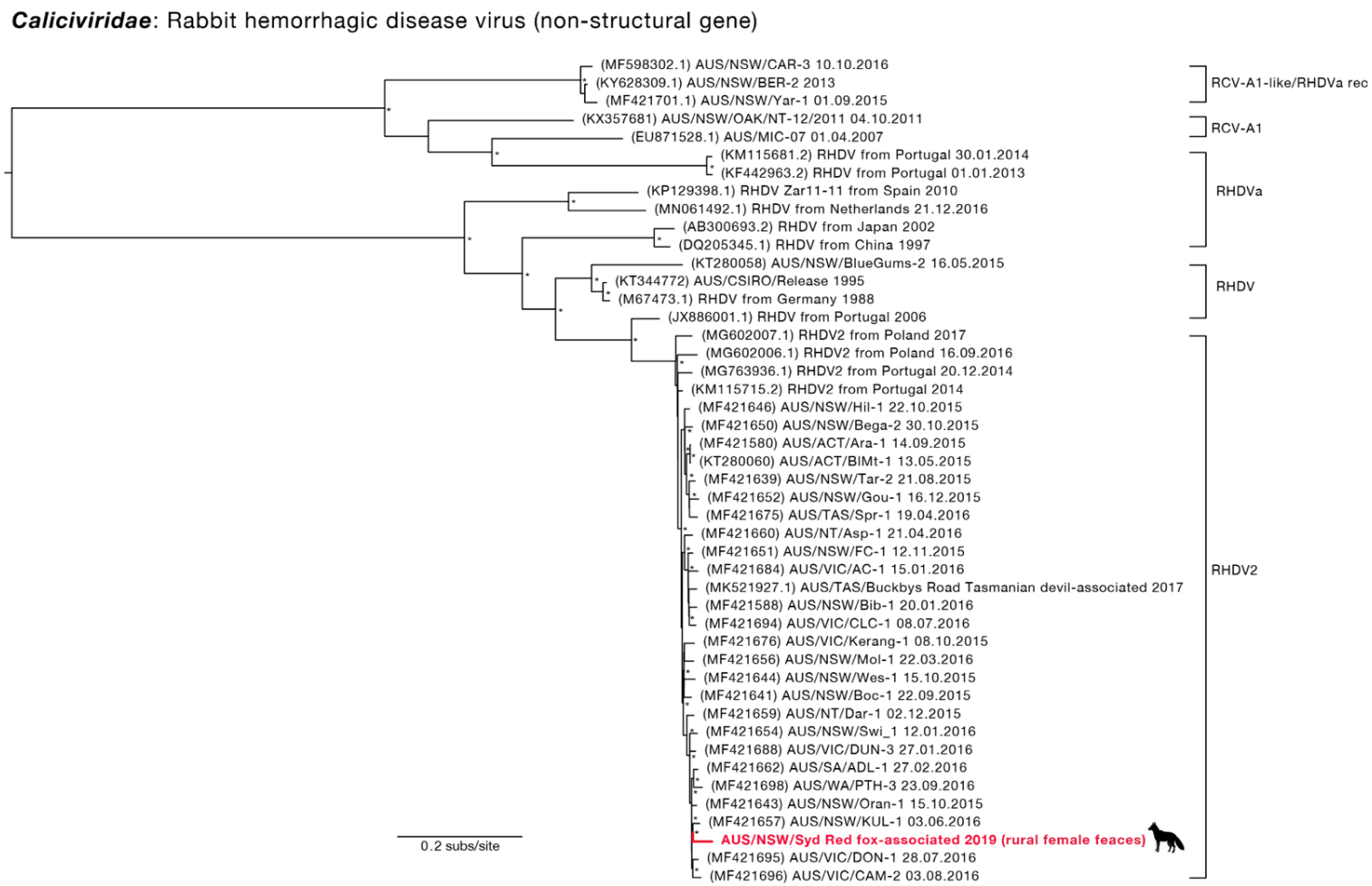

Figure 5. A maximum likelihood phylogenetic tree showing the topological position of RHDV2 in the red fox (bold, red text), in the context of its closest relatives. Major clades are labelled. All branches are scaled to the number of amino acid substitutions per site and trees were mid-point rooted for clarity only. An asterisk indicates node support of >70% bootstrap support.

Discussion

We have revealed that Sydney's red foxes, in both urban and rural environments, harbour a wide diversity of viruses, some of which are genetically similar to those that infect domestic pets and humans.

Discovery of novel viruses

We discovered viral transcripts with sequence similarity to the *Hepeviridae*, which causes hepatitis E in mammals. Hepatitis E can result in serious illness in humans and can be fatal for pregnant women or the immunocompromised (118). Hepatitis E virus has been isolated from various domestic and wild animals (119). Confirmed zoonotic cases include transmission to humans from domestic pigs, cats and wild rodents (119, 120). The red fox hepevirus found here exhibited the closest homology to hepevirus in freshwater mussels with 28.92% similarity. This virus is divergent from other hepeviruses found in foxes in the Netherlands, which are more closely related to hepatitis E virus in other mammals (73).

The fox astrovirus related transcript showed the greatest homology to astroviruses from domestic cats as well as from other foxes, humans and pigs. Astroviruses have a broad host range (121) and are frequently detected in the faeces of mammals, birds and humans with gastroenteritis (122, 123). Astroviruses have also been associated with other diseases and disorders such as shaking syndrome in minks (124), neurological disease in cattle (125) and encephalitis in humans (126). Some human astroviruses are more closely related to those in animals than to each other, suggesting that these viruses periodically emerge from zoonotic origins (127). The similarity of fox astroviruses to those found in cats indicates that these viruses have jumped hosts in the past and highlights the role that domestic pets and wildlife play in virus transmission.

Picobirnaviruses are found in humans and other mammals and are thought to be linked with gastroenteritis, however their role in disease remains unclear (128, 129). The picobirnavirus related transcript found here showed the greatest homology to a picobirnavirus found in diarrheic wolves (128). It is also similar to picobirnaviruses described as potentially zoonotic in humans with gastroenteritis (130). There is, however, evidence that suggests picobirnaviruses may actually be bacteriophages rather than eukaryote viruses (131).The virology of picobirnaviruses remains unclear.

We uncovered novel fox viruses within the *Picornaviridae* belonging to three distinct genera: kobuvirus, picodicistrovirus and picornavirus. The *Picornaviridae* are a large family that include viruses responsible for human disease such as hand, foot and mouth disease, polio, myocarditis, hepatitis A virus and rhinovirus (132). All viral sequences here showed greatest homology to viruses found in dogs. While we cannot conclude whether these viruses cause disease, it is worth noting that kobuviruses have been isolated from dogs and other mammals with diarrheic symptoms (133, 134) and there is evidence to suggest kobuviruses undergo frequent cross-species transmission (135). Additionally, the fox picornaviruses found here are closely related to sapeloviruses that cause encephalitis in domestic pigs (136-138).

Finally, we discovered rabbit haemorrhagic disease virus-2 (RHDV2) in fox faeces. Belonging to the *Caliciviridae* family, RHDV was released in Australia in 1995 following testing as a biological control agent for invasive rabbits. A variant of the disease, RHDV2, began circulating in Australia in 2015 and is presumed to be an incursion from Europe where it first emerged in 2010 (139). RHDV2 has now become the dominant strain circulating in Australia's wild rabbits (140). The virus identified here showed sequence homology to RHDV2 strains found in rabbits in New South Wales, Australia in 2015-2016. It can be assumed that Sydney foxes consume diseased rabbits and the virus is simply a gut contaminant, with no active RHDV2 replication in the fox host. However, serological assays would be needed to confirm whether the virus can replicate and therefore infect foxes.

It might be possible that RHDV2 found in foxes was the result of infected fly consumption while scavenging. RHDV can be transmitted by flies after contact with diseased rabbit carcasses and remain viable for up to 9 days (141). RHDV can also be excreted in fly faeces and regurgitate, which contains an adequate number of virions to infect rabbits (141). Indeed, flies may be important vectors for pathogen transmission for scavenging predators such as foxes.

The red fox virome across a land use gradient

Urbanisation influences pathogen exposure and prevalence in wildlife. For example, the prevalence of parvovirus increases with proximity to urban areas in grey foxes (*Urocyon cinereoargenteus*) in the US (142). In addition, dogs in urban areas in Brazil harboured

more tick-borne pathogens than rural dogs (143). It has also been shown that prevalence of West Nile virus in wild birds in the US increases with proximity to urban areas and human population density (144).

Despite the findings in the studies mentioned above, we found that overall abundance of viruses was highest in rural foxes. This result could indicate disease in some of these animals characterised by a compromised immune system and increased viral replication (145). Alternatively, this finding could indicate that foxes in highly urbanised areas experience reduced exposure to viruses by comparison with rural foxes. For example, exposure to canine distemper virus in red foxes has been shown to decrease in highly urbanised areas, which is thought to be due to reduction in movement corridors for wildlife and hence transmission pathways and virus vectors (146). Nevertheless, the risk of exposure to canine distemper virus increased in areas with more natural habitats (146). Urban green spaces or remnant forest may therefore be hotspots for pathogen transmission due to a greater convergence of urban wildlife (146).

Further, there is a possibility that urban living may reduce fox susceptibility to viral infection by positively influencing host immunity. For example, an abundance of rich food sources can increase nutritional intake, positively influencing overall health and condition and hence resistance to viral infections (147). Kit foxes (*Vulpes macrotis*) in urban areas in California show less nutritional stress, increased body condition and improved immune function when compared to foxes in a nearby nature reserve (148). In addition, Australian lace monitors (*Varanus varius*) that feed on human refuse experience improved body condition and reduced blood parasite infection compared to those that do not subsist on anthropogenic food waste (149). Since foxes in urban Sydney grow larger and are heavier than foxes in rural areas (95) there may be an advantage to consuming anthropogenic food sources for overall condition and pathogen resistance.

The virome of male and female red foxes

Across land use types, we found that female foxes harboured a higher abundance and diversity of viruses compared to male foxes. This finding could be explained by female foxes being more social than males (i.e. associating with cubs and other ‘helper females’) (97, 98), resulting in more frequent and varied opportunities for viral transmission. While this idea has not yet been supported by direct field observations, our finding of increased

viral abundance and diversity in female foxes suggests that host social behaviour may be an important factor influencing the virome and could play an important role in virus transmission and emergence.

There may be multiple co-occurring factors that could affect viral infection in Sydney's foxes. Additional assessments of habitat structures, fox densities, movement behaviours and social dynamics in urban and rural areas in the Greater Sydney region will help to elucidate those factors. An obvious extension to this work would be to examine the virome of foxes across a more comprehensive urban-rural gradient, including foxes from more isolated, bushland regions. Such a study may help us to understand how pathogen prevalence and transmission differs between isolated, natural landscapes and human dominated regions, and how introduced species may contribute to disease prevalence in these environments.

Conclusion

As human encroachment on wild environments increases and wild animals continue to adapt to urban areas, the frequency of human-wildlife interactions intensify. The effects of urbanisation on pathogens harboured by wildlife may have unexpected consequences for human and domestic animal health. We are unable to say definitively whether the viruses identified here cause disease in foxes. Nevertheless, foxes living in Greater Sydney carry viruses that share genetic similarity to those found in domestic animals and humans. Our findings indicate that foxes may be reservoirs for viral pathogens with zoonotic potential.

Chapter III: Final discussion and future directions

Red foxes in the Greater Sydney region host a wide diversity of viruses. Some of these viruses are associated with diseases of zoonotic potential. Aside from viruses, foxes carry other microbes and parasites of veterinary and medical significance (13, 33, 47, 55, 57, 67, 150). It should be highlighted that viruses made up only a small proportion of the meta-transcriptome described in this study. In fact, the overwhelming majority of taxa in fox faeces were bacteria.

A thorough analysis of the entire fox 'infectome' (i.e. viruses, bacteria, parasites and fungi) was beyond the scope of this study. However, a comprehensive analysis of the bacteria and other eukaryotic organisms in both urban and rural foxes would assist to determine their role in harbouring and transmitting disease. Indeed, the meta-transcriptome data generated here could be used to reveal bacterial, parasitic and fungal communities, as well as the expression of host and pathogen genes associated with immunity and antimicrobial resistance, respectively. Both of these avenues of research are worthy of further exploration, as discussed further below.

The effect of urbanisation on microbial communities

Urbanisation can affect microbe composition (142, 146, 151, 152), however, disease dynamics in urban wildlife compared to wild environments are not well understood. Human activities in urban settings present a plausible means of disruption to the normal transmission pathways and networks for microbes and their hosts that are known to occur in wild environments. The data generated in the course of this study could also be used to reveal any potential effects of urbanisation on the fox microbiome.

Microbial diversity is influenced by many factors including host phylogeny, diet, habitat, pollutants and other environmental conditions (153-158). Gut microbial diversity is positively associated with overall host health (159) and may have important functions in determining host immunity, pathogen resistance, digestion, nutrient absorption and even behaviour (160-163).

While the effect of urbanisation on microbial diversity in wild mammals has not yet been explored, urbanisation has been associated with significant changes in the diversity of gut microbes in wild passerine birds. On the one hand, reduced microbial diversity has been observed in urban house sparrows when compared to their rural counterparts (164). On

the other, the opposite effect was observed in white-crowned sparrows (165). Whether such changes result in harm to these animals remains untested. However, such notably opposing results highlight the need for further exploration of the effects of urbanisation on microbes and their hosts.

Birds are highly mobile. The mechanisms that determine their gut microbial composition may not be analogous to ground dwelling mammals. Regardless, the significant effects of urbanisation on bird microbiomes suggests that similar observations would be expected in foxes, even if the relevant mechanisms differ. Exploring the red fox microbiome across an urban-rural gradient would test this hypothesis. Any differences would shed light on the health status of foxes in each category, the bacterial pathogens that they carry and how urban living may influence fox ecology.

Antimicrobial resistance in the red fox

Meta-transcriptomics can reveal the expression of microbial genes such as those associated with antimicrobial resistance (AMR). Antimicrobials play an important role in the treatment of bacterial infection in humans and domestic animals. However, due to the widespread and ongoing use of antimicrobial products, pathogenic bacteria have developed resistance. The evolution of AMR has led to the rise of multidrug resistant bacteria or ‘superbugs’ (166). There is now evidence that AMR bacteria have spread to wild animals that have never been treated with, or even encountered, pharmaceutical antibiotics (167, 168).

Human activity is closely linked to AMR prevalence and may offer prime conditions for the evolution and transmission of AMR genes. The Centre for Disease Control and Prevention in the US estimate that approximately 2 million people per year are infected with AMR bacteria and around 23,000 subsequently die (169).

Proximity and exposure to human activity is thought to be the best predictor of whether wild animals harbour AMR bacteria (170). Urban centres are a hub of human activity with antibiotic wastes and AMR bacteria in high concentration. In keeping with these observations, two studies in Europe comparing AMR bacteria in foxes from urban and rural regions found that foxes living closer to more densely populated areas had higher prevalence of AMR (150, 171). Therefore, foxes harbouring AMR bacteria provide a plausible vector for the dispersal of AMR throughout urban areas and rural environments.

Due to their varied and opportunistic diet, foxes are likely to eat human refuse containing antibiotics or AMR bacteria as well as other animals that are likely to harbour AMR bacteria (e.g. birds and rodents (171)). The amplification of AMR bacteria up the food chain potentially explains their prevalence and the increasing occurrence of multidrug resistance in predators (172-174). In the absence of dingos, foxes have become significant predators within Australia's disturbed, urban ecosystems. This amplification effect may thus contribute to the prevalence of resistance genes in this species, particularly in urban regions. Further AMR studies in foxes, which so far have been limited, would assist in describing the role that urban predators have to play in AMR bacteria transmission.

Moreover, given their position towards the top of the urban food chain, detecting AMR bacteria prevalence and diversity in Australia's urban foxes may assist in understanding the overall prevalence of AMR bacteria in urban food webs.

Edge effects on pathogen diversity and transmission

Urban environments can support high numbers of wild carnivores, such as foxes, coyotes, and raccoons (1), as well as other urban adapted species. Nevertheless, biodiversity in urban areas is often reduced due to the exclusion of wildlife that are not suited to urban living or are outcompeted by urban adapters (175). Urban fringes or edges between rural and urban or peri-urban environments likely support a greater diversity of wildlife than urban interiors (176). For example, arboreal mammals in southern NSW occur rarely in highly urbanised interiors (except the urban adapted brushtail possum) and increase significantly in abundance and diversity towards bordering forest edges (177). Similarly, bird species richness is generally higher in grassland and shrubland habitats bordering major cities, when compared to adjacent urban areas (178). Furthermore, many meso-predators can persist towards urban boundaries (179) with some even preferring forest edges to forest interiors (180). These urban-forest interfaces are thus likely to provide multiple pathways for pathogen transmission between urban and rural wild animals, as well as humans and domestic species in neighbouring urban areas (2, 181).

Chapter II reveals a higher viral abundance in rural foxes compared to urban individuals. Due to the difficulty in obtaining foxes from isolated bushland areas, most of the rural zones delineated in this study bordered urban areas – i.e. the types of 'urban fringes' just referred to.

Home ranges of urban foxes in Australia are small (less than 1km^2) (86) suggesting that travel between urban and rural habitat is unlikely. However, fox behaviour is highly plastic. After an outbreak of sarcoptic mange in the city of Bristol in the UK, which resulted in high fox mortality, surviving foxes home ranges increased by 11-fold (182). This change in range size was not associated with food availability or any other limiting resource or dispersal behaviour, but was instead attributed to territory expansion by foxes moving into deceased neighbours' territories. Territory expansions likely increased until foxes encountered a dominant individual and could not expand further (182).

At urban borders, particularly those where fox control is sporadically implemented, home range plasticity may mean foxes have the capacity to move in and out of more urbanised areas. Another direction for future research would be to assess fox ranging behaviour across an urban to rural gradient in Greater Sydney. This would provide important data on fox movement dynamics, and shed light on how sporadic control operations may affect fox ranging behaviour and impact disease transmission networks.

Conclusion

Despite their large presence and plausible role in pathogen prevalence in urban areas, the pathogen load of invasive foxes in the Greater Sydney region has received little attention. The findings in this thesis raise additional avenues for future research concerning the effects of these invasive predators in Australia's urban areas. Particular focus could be fruitfully directed at revealing the entire infectome of urban foxes. Additionally, it would be worthwhile to analyse the relationship between the infectome and landscapes along an urban-rural gradient for example, by using proxies such as the amount of green space (parks, gardens and remnant habitat) and grey space (buildings and other human infrastructure). Such analysis would likely help in identifying potential hotspots for pathogen transmission.

Disease surveillance of wildlife within urban landscapes is necessary for detecting pathogen emergence. Utilising the innovative meta-transcriptomics approach described here will help us gain a better understanding of pathogen genetic diversity, evolutionary histories, the complexities of inter and intra-species transmission, and the circumstances which lead to emerging infectious diseases.

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