Co-evolution of Rabbits and the Rabbit Haemorrhagic Disease Virus (RHDV) in Australia

Amy Iannella

School of Biological Sciences

University of Adelaide

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Supervisory panel: Associate Professor Phillip Cassey, Dr Nina Schwensow and Dr David Peacock

Table of Contents

Abstract

The European rabbit is a keystone prey species in its native range on the Iberian Peninsula, where it is under threat from two viral diseases - myxomatosis and the rabbit haemorrhagic disease virus (RHDV). However, in its alien range the rabbit is considered one of the most damaging pest species, due to overgrazing of pastures and native vegetation. In these areas myxomatosis and RHDV have been deployed as biocontrols for landscape-scale rabbit management. Despite the initial success of viral biocontrols, increases in rabbit abundance were observed between 2003 and 2015, and evolving rabbit genetic resistance to RHDV was proposed as a key cause. Although substantial investment has been made in offsetting such resistance, through the development and introduction of new RHDV strains, the existing co-evolutionary dynamic between rabbits and RHDV in Australia is poorly understood. My thesis uses next-generation sequencing technology to explore the co-evolution of rabbits and RHDV through three approaches, presented as three publication-style manuscripts.

In Chapter 2, I pilot the use of blowfly vectors to monitor spatial and temporal variation in RHDV strains. I find that wind-oriented fly traps provide improved efficiency and viral detection rates that exceed previously used rabbit carcass searches. Shotgun sequencing of RHDV capsid amplicons indicated multiple co-circulating local RHDV variants with evidence of recombination between them. This implies that intraspecific competition may play a substantial role in the direction of RHDV evolution, in addition to host resistance.

In Chapter 3, I examine the underlying genetic structure of Australia's rabbit population through a genome-wide selection of SNP loci produced through reduced representation sequencing. I find strong support for three geographically widespread rabbit lineages, as well as three individual sites with strong local differentiation. This genetic structuring is consistent with an invasion history of multiple introductions, rather than the previously assumed single primary invasion front, and may contribute to geographic variance in RHDV resistance.

In Chapter 4, I combine long-term capture-mark-recapture data from a single rabbit population with a SNP-based pedigree using reduced representation sequencing. I find evidence of socially structured polygynandry with male-biased dispersal and an unexpectedly high rate of breeding outside warren-based social groups. I also examine the influence on offspring survival of warren size, birth dates and maternal antibodies to RHDV and myxomatosis.

This thesis contributes to our understanding of rabbit and RHDV co-evolution by validating a more effective RHDV monitoring tool, characterising the underlying genetic variation in both host and pathogen, and analysing the pressures that drive evolution of this host-pathogen system. These insights provide the building blocks for further research to understand the

mechanisms of genetic resistance to RHDV in rabbits, the extent of influence of resistance on rabbit fitness and abundance, and the impacts of current and future biocontrol activities.

Declaration Statement

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 a 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.

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Chapter 1 – Background

The European Rabbit

The European rabbit, *Oryctolagus cuniculus*, is native to Spain, Portugal, southern France and northern Africa but is now widely spread, especially across western Europe and the Pacific islands (Smith and Boyer 2008). Known for its extremely high fecundity, a single adult female can produce over 15 kittens in a year, depending on climate (Gilbert *et al.* 1987; Tablado *et al.* 2009) and social rank (von Holst *et al.* 1999). Rabbits live in social groups which may either seek shelter beneath dense shrubs and rock formations, or construct warrens, which provide shelter from both predators and the elements (White *et al.* 2003). Rabbits experience high juvenile mortality, while adults typically live two to three years and have been recorded to reach seven years of age (Peacock and Sinclair 2009; von Holst *et al.* 1999). Across many areas of Europe rabbits function as a keystone prey species within the ecosystems, forming the primary food source for predators such as the endangered Iberian lynx (*Lynx pardinus*) and Spanish imperial eagle (*Aquila adalberti*) (Delibes-Mateos *et al.* 2007; Palomares *et al.* 2001).

The European rabbit was primarily introduced to Australia in the early-mid 19th century by settlers as a source of meat and fur and for sport hunting. Rabbits rapidly established large and increasingly dispersed populations, which became recognised as agricultural pests (Cooke 2007). Rabbits increase the grazing pressure on pastures, significantly decreasing their carrying capacity for livestock and causing an estimated annual loss to the livestock industries of AUD$2073 million (2011 dollars) prior to the introduction of biocontrols (Cooke *et al.* 2013). Additionally, rabbits have caused widespread ecological devastation through overgrazing in competition with native herbivores. They also provide a food resource sufficient to maintain high populations of introduced predators, which severely inhibit many native fauna species. The severity of these impacts on native fauna is demonstrated by the findings of Pedler *et al.* (2016) who show dramatic increases in the extent of occurrence of the threatened dusky hopping mouse (*Notomys fuscus*), plains mouse (*Pseudomys australis*) and crest-tailed mulgara (*Dasycercus cristicauda*) following sustained rabbit biocontrol. Rabbits reduce vegetation diversity through selective overgrazing of palatable species, particularly young shoots and seedlings, which effectively prohibits recruitment of these species and undermines ecosystems in which such species play a significant ecological role (Bird *et al.* 2012; Cooke and Mutze 2018; Mutze *et al.* 2016b). Furthermore, rabbits are known to promote invasive weeds through selective grazing of palatable natives and producing soil conditions that favour exotic species adapted to rabbit presence (disturbance, higher soil nutrients, seed dispersal in dung) (Cooke 2012).

As a species with such high fecundity, which impacts native vegetation at densities as low as 0.5 ha^{-1} (Bird *et al.* 2012), rabbit abundance needs to be driven very low to prevent rapid

resurgence and to effectively mitigate their environmental impacts. Therefore, a combination of control strategies such as shooting, poisoning with sodium fluoroacetate (1080) on oat bait, destruction of warrens by 'ripping' with a bulldozer and then fumigation of reopened burrows, have traditionally been employed in Australia (Williams and Moore 1995; Williams *et al.* 1995).

For a costly pest that was in plague numbers across vast (largely uninhabited) areas of Australia, where conventional control methods are uneconomical, novel biocontrol solutions were required. Myxomatosis, a benign disease of *Sylvilagus minensis,* which is lethal in *Oryctolagus cuniculus*, was introduced to Australia in 1950 causing rabbit declines of up to 99% (Fenner *et al.* 1953). Myxomatosis is spread primarily by mosquitoes, particularly *Anopheles annulipes* and *Culex annulirostris*, and the introduced European rabbit flea (*Spilopsyllus cuniculi*), as well as the Spanish rabbit flea (*Xenopsylla cunicularis*), which was deliberately introduced as a vector adapted to the hot dry regions in which mosquitoes are unreliable vectors. Within a decade the intense selective pressure caused by myxomatosis resulted in increased genetic resistance throughout the rabbit population, which began to gradually re-proliferate (Kerr 2012).

Introducing RHDV

Rabbit haemorrhagic disease (RHD) is caused by the rabbit haemorrhagic disease viruses (RHDV) of the family Caliciviridae, which has a single RNA strand that was sequenced in 1991 (Meyers *et al.* 1991). Infection can be caused by as few as 10 viral particles (Gehrmann and Kretzschmar 1991). The viral disease causes inflammation and cell death in the liver, clotting of the blood, which can cause haemorrhages, and respiratory and cardiovascular failure, usually resulting in death within 36 hours. External symptoms can include fever, shortness of breath, lethargy and epistaxis (bloody discharge from the nose) but are often not apparent (Hukowska-Szematowicz *et al.* 2013). RHD was first recorded in China in 1984 and was then rapidly found throughout much of the world, likely spread through international live rabbit trade (Cooke and Fenner 2002). Two further clades of RHDV later emerged in Europe, known as RHDVa and RHDV2 (Capucci *et al.* 1998; Le Gall-Reculé *et al.* 2013). A summary of known RHDV strains is presented in Appendix 1. The original clade, known as RHDV1, was the only clade known in Australia until 2014 and is the focus of the research presented in this thesis. When referring to all clades collectively in this thesis I will use the term RHDV.

Declining rabbit numbers in Europe have been largely attributed to the combination of myxomatosis and RHD; and are strongly implicated in the decline of predators such as the Iberian lynx and Spanish imperial eagle (Delibes-Mateos *et al.* 2007; Palomares *et al.* 2001). Noting the impact of RHDV1 in arid Spain in 1988, Dr Brian Cooke proposed the introduction of the virus as a bio-control in Australia. Tests under quarantine in 1991 confirmed the high lethality and species-specificity of the virus, and in 1995 a quarantined field trial followed on

Wardang Island, in the Spencer Gulf of South Australia (Cooke 2014). Despite precautions the virus spread to the mainland, likely by way of insect vectors, and became naturalised (McColl *et al.* 2002a). The initial outbreak of RHD had varying impact with up to 95% population reduction; the greatest impacts were generally recorded in more arid regions (Cooke and Fenner 2002; Mutze *et al.* 2008; Mutze *et al.* 1998). Since then RHDV1 has produced natural outbreaks in winter or spring every 1-2 years, annually in many areas, maintaining a significant level of rabbit population suppression. However, evidence between 2003 and 2015 increasingly suggested that rabbit numbers were recovering in many areas due, at least in part, to emerging genetic resistance (Elsworth *et al.* 2012; Mutze *et al.* 2014a).

RHDV biological transmission and rabbit immunity

RHDV is thought to be transmitted primarily through oral, nasal or conjunctival contact with an infected rabbit or contaminated material (Asgari *et al.* 1998). Biting insects may also facilitate transmission (Cooke 2007). Virus particles are shed by infected rabbits in large quantities within urine and faeces, and are also present in skin, fur, blood and secretions (Nystrom *et al.* 2011); and in carcasses for at least 26 days after death (Henning *et al.* 2005; McColl *et al.* 2002b; Mitro and Krauss 1993). As a result, it appears that flies, contaminated through contact with infected rabbits, carcasses or faeces, disperse viable virus over large distances, transmitting the virus via their regurgitated or defecated 'fly spots', which may be ingested via grass or during grooming (Asgari *et al.* 1998). Schwensow *et al.* (2014) showed that RHDV1 outbreak strains from consecutive years are most often not each other's nearest relative, indicating that this landscape-scale transmission plays a larger role in initiating outbreaks than any virus particles maintained within a population between seasons.

Studies of RHDV1 and the related human Norovirus have shown that these viruses bind to histo-blood group antigens (HBGAs), which are present on the surface of human red blood cells (causing the differential blood types), but in saliva, epithelial cells and the mucous of the digestive tract of rabbits. Binding to these antigens facilitates cell entry for the virus, but different virus strains appear to bind with some specificity toward different antigen types, conferring a level of resistance to infection at low dosages for individuals with HBGA types; to which a given virus strain binds only weakly (Nyström *et al.* 2011). Expression of the HBGAs in rabbits appears to be controlled by the Sec1 gene, with low expression Sec1 alleles correlating with RHDV1 outbreak survival (Nyström *et al.* 2015).

Young rabbits are known to possess considerable resilience to RHDV1, decreasing until an adult level of susceptibility is reached at roughly three months of age (Xu and Chen 1989). Ruvoën-Clouet *et al.* (2000) showed that the HBG antigen H is required for binding with RHDV and is not present in kittens until three weeks of age, gaining full expression at around 10 weeks old. This lack of HBGA secretion may therefore contribute to juvenile

resistance to infection, although it cannot explain the extremely low level of liver infection that results from intramuscular inoculation of four-week old rabbits with RHDV1 (Ferreira *et al.* 2004).

Rabbits that recover from RHD produce antibodies against the virus, which apparently confer lifelong protection from that serotype given repeated exposure to the disease, which boosts antibody titres. The IgG antibodies pass across the placenta to embryos, granting a level of protection until about 12 weeks of age, depending on the mother's antibody titre. Although kittens with maternal antibodies are not protected from infection, disease severity is reduced and chance of survival increased (Cooke *et al.* 2000; Robinson *et al.* 2002).

RHDV epidemiology

As a result of kitten resilience to RHDV1, outbreak timing has a significant impact on the effectiveness of the disease. Outbreaks during the middle to the end of a breeding season infect a large number of resistant kittens, which survive and develop lifelong immunity, contributing to the breeding population of future years. In contrast, RHD outbreaks at other times of the year do not infect the majority of new kittens, permitting greater initial kitten survival, but leaving these individuals susceptible to outbreaks in further years once juvenile resistance has waned, often before the individual has a chance to breed (Mutze *et al.* 2008; Wells *et al.* 2015). Outbreak timing is apparently dependent on climate, insect vector activity and the density of susceptible individuals. RHDV1 outbreaks have typically settled into an annual pattern, occurring during winter in arid areas (early breeding season) and spring in cooler wetter zones (mid breeding season) (Henzell *et al.* 2002; Mutze *et al.* 2002; Wells *et al.* 2015). The differential timing of RHDV outbreaks may thus play a role in the increased effectiveness of the virus in arid zones. Calvete *et al.* (2006) and Calvete (2006) also note that in more productive habitats rabbits are better able to maintain numbers, despite RHDV, due to increased breeding output and the lower average age of RHDV exposure. The timing of RHD outbreaks is not static and may be influenced by the co-evolution of rabbits and RHDV. Mutze *et al.* (2014b) found that over time outbreaks at Turretfield in South Australia were becoming longer and occurring later in the season. Analysis of changes in carcass data lead them to conclude that the virus was increasing in infectivity towards kittens, for whom the mortality rate is low, and hence seroconversion and immunity was increasing, contributing to increased local (and potentially national) abundance.

In semi-arid areas, the heavy reduction in rabbit numbers driven by RHDV1 during winter appears to reduce the population below the threshold required for myxomatosis to spread effectively, such that myxomatosis outbreaks have begun occurring when rabbit numbers peak in autumn, rather than the spring/summer outbreaks common prior to RHDV1. In this way the effectiveness of both diseases is being maintained and populations have remained lower than in the presence of a single pathogen. In some areas of higher rainfall, however,

myxomatosis outbreaks occur predominantly in spring following rabbit breeding, at a similar time to outbreaks of RHDV1. It appears that in these areas the two diseases may compete for the same subadult hosts such that the presence of a second pathogen does little to further reduce rabbit density (Bruce *et al.* 2004; Mutze *et al.* 2002). A synergistic effect between RHDV and myxomatosis has been suggested. Marchandeau *et al.* (2004) found that rabbits seropositive to either virus were more likely to be seropositive to the other, while modelling by Barnett *et al.* (2018) showed that prior exposure to myxomatosis reduced individual survival in RHDV outbreaks by 10%. However, modelling by Wells *et al.* (2015) presents a more complex picture. Wells *et al.* found that myxomatosis recovery rates had a substantial impact on RHD fatality and on probability of population persistence, but these effects depended heavily on outbreak timing. The interactions between RHDV, myxomatosis, rabbit demographics and climate appear to be complex and further research is required to better understand this dynamic system.

Further to the impacts of outbreak timing and interactions with myxomatosis, RHDV is less effective in cooler regions of higher rainfall due to the presence of a benign calicivirus strain, RCV-A1, in these areas (Cooke *et al.* 2018; Liu *et al.* 2014). RCV-A1 is a close relative of RHDV and was present in Australia prior to the introduction of RHDV1 (Cooke *et al.* 2002; Strive *et al.* 2009). It does not cause detectable disease symptoms but does cause rabbits to produce antibodies, which partially protect against RHDV1 infection (Cooke *et al.* 2018) and reduce mortality by around 50% in both field and laboratory studies (Mutze *et al.* 2010; Strive *et al.* 2010). The presence of RCV-A1 may therefore be an underlying cause of reduced RHDV effectiveness in cooler, wetter areas (Liu *et al.* 2014), with flow-on effects on the interaction between RHDV and myxomatosis.

RHDV and the future: Co-evolution

By comparing infection rates of domestic and wild rabbits with low doses of the RHDV1 Czech 351 strain, Elsworth *et al.* (2012) found that wild rabbits from areas of moderate rainfall have become significantly less prone to infection, and that infection in these rabbits tends to be more protracted, which may increase their chances of survival. This implies that wild rabbit populations in similar areas of Australia, and likely elsewhere, have begun to develop resistance to RHDV1, which could lead to steady increases in these rabbit populations over time, as happened in the years following the initial introduction of myxomatosis. Schwensow *et al.* (2017a) found that, despite historical population bottlenecks, Australian rabbits exhibit substantial variation in major histocompatibility complex (MHC) class I immune genes. This variation is likely to be a response to RHDV-driven frequency-dependent selection, suggesting that this important group of immune genes plays a key role in rabbit immune response to RHD. Schwensow *et al.* (2017b) were also able to identify 72 genetic loci with 133 associated genes that appear to correlate with RHD resistance in rabbits, however, further research is required to identify the most

important genes responsible, and to determine the underlying mechanisms by which they cause resistance.

Van Valen's famous Red Queen Hypothesis (Van Valen 1974) describes the process of co-evolution by natural selection in interacting organisms, such as the host-pathogen interaction between rabbits and RHDV. When one organism evolves an advantage over the other (i.e., rabbits gaining resistance to infection through a particular gene) the selective pressure on the disadvantaged organism is increased such that it evolves mechanisms to gain advantage over the other, creating an eternal "arms race". In this way RHDV may be evolving mechanisms to circumvent resistance in Australian rabbits. Indeed, Domingo and Holland (1997) highlighted that RNA viruses are particularly adept at mutating and evolving in this way and Kovaliski *et al.* (2014) have shown that RHDV in Australia is evolving much more rapidly than its foreign counterparts, at a rate of ~4.7×10^{-3} substitutions per site per year.

The co-evolution process has previously been observed between rabbits and myxomatosis. The extreme mortality of rabbits following the introduction of myxomatosis to Australia applied enormous selection pressure on the rabbits, which developed resistance to the disease, and thus to the virus which cannot replicate and spread in dead rabbits. The apparent equilibrium that has resulted consists of myxomatosis strains that cause protracted disease with moderate mortality in resistant rabbits, such that the rabbits survive in a contagious state for a maximum period (Fenner and Marshall 1957; Kerr 2012).

That RHDV is responding to evolutionary pressures is clear, with new variations recorded globally every year (Hukowska-Szematowicz *et al.* 2013; Kovaliski *et al.* 2014; Schwensow *et al.* 2014; Wang *et al.* 2012) and recombination events resulting in hybrid strains have also been observed (Abrantes *et al.* 2008; Forrester *et al.* 2008; Hu *et al.* 2016; Mahar *et al.* 2018b). In the last two decades, two distinct new clades of RHDV have emerged in Europe, labelled RHDVa and RHDV2 (Capucci *et al.* 1998; Le Gall-Reculé *et al.* 2013). These strains have each in turn become dominant in both domestic and wild populations (Lavazza *et al.* 2004; Lopes *et al.* 2014) and display differing virulence characteristics to the originally identified strains. In particular, RHDV2 has been shown to affect young and vaccinated rabbits (Dalton *et al.* 2012), wild rabbits with antibodies to RHDV1 (Peacock *et al.* 2017), as well as hares (Camarda *et al.* 2014; Hall *et al.* 2017; Puggioni *et al.* 2013), all of which were typically resistant to the original RHDV1 strain.

In 2014 a recombinant strain of RHDVa, with likely Chinese origin, was identified in Australia near Sydney where it persists locally (Mahar *et al.* 2018b), while a strain of RHDV2 was detected in the Canberra region in 2015 (Hall *et al.* 2015). RHDV2 has rapidly spread throughout Australia, largely replacing the endemic RHDV1 strain (Mahar *et al.* 2018a). It has been shown to overcome immunity to RHDV1 (Peacock *et al.* 2017) and cause a substantial reduction in rabbit numbers (Mutze *et al.* 2018), as well as recombine with other

strains (Mahar *et al.* 2018b). A second strain of RHDVa originating in Korea (known as K5) was shown to have increased effectiveness against rabbits with RCV-A1 antibodies (RHD-Boost 2014), and was deliberately released as a biocontrol agent in 2017 (World Organisation of Animal Health 2017). The competitive fitness of this strain in the Australian environment, and its impact on RHDV epidemiology, remains to be determined.

At present there is a lack of understanding of the ecological and evolutionary processes involved in the emergence of new virus variants. The long-term impact of rabbit genetic resistance to RHDV will depend on whether the trending direction and rate of RHDV evolution maintains infection and mortality rates against the evolution of resistance in wild rabbits. Insight into the selective pressures under which new RHDV variants develop, the way they are transmitted between populations, and whether there is significant competition for rabbit hosts, will be of benefit in understanding the likely directions of RHDV in the future. The research presented here begins the process of understanding these drivers of RHDV evolution.

Aims of this research

In order to generate accurate population models and inform Australian rabbit management decisions we must first understand the key drivers of rabbit population dynamics and the evolutionary pressures that are causing these dynamics to change. Recent advances in reduced representation sequencing protocols presented an opportunity to investigate the co-evolution of the feral European rabbit and RHDV1 in Australia and to develop an understanding of this rapidly changing driver of rabbit population dynamics.

In Chapter 2 I aim to improve RHDV monitoring capabilities by assessing the use of fly (Dipteran) vectors to monitor spatial and temporal variation in RHDV strains. I use trapped flies to examine the spatio-temporal variation of RHDV1 in South Australia and examine the ramifications of this variation for inter-strain competitive adaptation.

In Chapter 3 I aim to quantify the genetic variability of Australian rabbits in order to assess the likelihood of national-scale genetic structure contributing to variation in RHDV susceptibility and the appropriate scale of rabbit management units to minimise re-invasion.

In Chapter 4 I aim to identify the drivers of rabbit reproductive output and survival, and hence abundance, by targeting a single South Australian population for detailed pedigree analysis. I also aim to clarify rabbit breeding and dispersal strategies, which may impact on the spread of genetic RHDV resistance factors within and between populations.

Finally, In Chapter 5 I evaluate the ramifications of my findings for rabbit-RHDV coevolution and for population management strategies. In this chapter I aim to identify useful avenues of ongoing research to further understand this environmentally and economically important evolutionary system.

Chapter 2 – Monitoring RHDV evolution

Statement of Authorship

Title of Paper	Vector-based monitoring of fly-borne disease: An overdue implementation in rabbit haemorrhagic disease virus (RHDV)
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Principal Author

Name of Principal Author (Candidate)	Amy Iannella		
Contribution to the Paper	Helped to manufacture fly traps. Collected and identified fly samples, performed rabbit carcass searches and field necropsies. Performed RNA extraction, transcription and amplification. Performed sequence data filtering, alignment and assembly. Performed data modelling and analysis, interpreted outputs and wrote manuscript.		
Overall percentage (%)	85%		
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.		
Signature		Date	6 Nov 2018

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.

Name of Co-Author	David Peacock		
Contribution to the Paper	Helped to manufacture fly traps and collect field samples. Helped in data interpretation, manuscript evaluation and editing		
Signature		Date	18 December 2018

Name of Co-Author	Phillip Cassey		
Contribution to the Paper	Supervised development of work, provided analysis advice, helped to evaluate and edit the manuscript.		
Signature		Date	18-December-2018

Please cut and paste additional co-author panels here as required.

Name of Co-Author	Nina Schwensow		
Contribution to the Paper	Supervised development of work, provided laboratory and bioinformatic advice, helped in data interpretation, manuscript evaluation and editing		
Signature		Date	14/12/2018

Vector-based monitoring of fly-borne disease: An overdue implementation for rabbit haemorrhagic disease virus (RHDV)

Abstract

Necrophagic flies are capable of spreading numerous pathogens, including rabbit haemorrhagic disease virus (RHDV). In this study we used the RHDV-rabbit host-pathogen system to demonstrate the benefits of using fly vectors for landscape-scale virus monitoring. We found that wind-oriented fly traps provided improved detection rate and efficiency over previously used carcass searches and were less reliant on researcher expertise and host density. Flies carrying RHDV were detected before and after rabbit carcasses and at locations where carcasses were not located. While multiple RHDV variants co-circulated in flies during each outbreak season, no site had more than one variant in rabbit carcasses, highlighting the important role of infectivity in RHDV intra-strain competition and evolution. Monitoring RHDV in flies provides improved baseline understanding of circulating strain variation and inter-strain competition, enabling improved monitoring and assessment of the impact of new strains.

Introduction

Monitoring the evolution and spread of viral diseases is critical for managing and assessing their impacts on wildlife, livestock and pest animals; particularly where they are, or may become, zoonotic. Carrion- and manure-visiting flies are capable of spreading several pathogens, including highly pathogenic avian influenza (Sawabe *et al.* 2006), Newcastle disease virus (Barin *et al.* 2010), enterotoxigenic *Escherichia coli* and *Vibrio cholerae* (Echeverria *et al.* 1983), and rabbit haemorrhagic disease virus (RHDV) (Asgari *et al.* 1998). As vectors, flies provide a unique opportunity for monitoring the prevalence and spread of these viruses. In this study we demonstrate the benefits of using fly vectors for virus monitoring using the RHDV-rabbit host-pathogen system.

Rabbit haemorrhagic disease virus has been a driving force behind rabbit mortality for the past two decades. RHDV has been heralded as a disaster in Europe where the European rabbit (*Oryctolagus cuniculus*) is a keystone native species and for the rabbit farms of countries like China, and a boon in Australia and New Zealand where rabbits are invasive and highly destructive. In Australia the introduced European rabbit causes widespread environmental (e.g., Bird *et al.* 2012; Mutze *et al.* 2016a; Mutze *et al.* 2016b) and agricultural damage (McLeod 2016). The enormous impact of this pest species prompted the importation of RHDV as a potential biological control agent. When the virus escaped from quarantine facilities on Wardang Island (South Australia) in September 1995 it spread rapidly to the mainland and across vast swathes of country (Kovaliski 1998). Fly vectors have been strongly implicated in this escape and rapid spread, with RHD transmission to rabbits

experimentally demonstrated in *Calliphora dubia* and *Musca vetustissima*, and successful detection of the viral RNA in *Chrysomya*, *Lucilia*, *Hydrotaea*, *Hybopygia* and *Sarcophaga* (Asgari *et al.* 1998; Barratt *et al.* 1998; McColl *et al.* 2002a). Flies become mechanical vectors of the virus when they visit infected rabbit carcasses, carrying viable virus for at least 20 days (Henning *et al.* 2005; McColl *et al.* 2002b).

Existing monitoring efforts for RHDV outbreaks in Australia are opportunistic, relying on spontaneous landholder reporting through which tissue samples can occasionally be solicited. Manual carcass searches are labour intensive and reliant on the observational capabilities and experience of searchers who are familiar with local rabbit warrens. Not all rabbits die above ground (Mutze *et al.* 1998), and these can often be quickly taken by scavengers, making visual searches unreliable where rabbit densities are low, or rabbits are inhabiting difficult terrain. A simpler monitoring technique that can be scaled up to numerous sites and implemented rapidly by inexperienced volunteers and in areas with lower rabbit densities is therefore desirable. Vector-based monitoring has been used internationally for surveillance of mosquito-borne viruses, such as West Nile Virus (Andreadis *et al.* 2001; Ochieng *et al.* 2013), Ross River and Barmah Forest Viruses (Hall-Mendelin *et al.* 2010), and for Bluetongue Virus in midges (Bishop *et al.* 2006) but has typically been overlooked for fly-borne diseases such as RHDV.

In 1985 Vogt *et al.* described a simple, cost-effective, wind-oriented fly trap, which is capable of trapping large numbers of several fly species, including those implicated in the spread of RHDV. In 1998 Asgari *et al.* used these traps to collect RHDV carrying flies and were able to detect the presence of the virus using RT-PCR both during an outbreak and in the month prior to and after the outbreak. Although their study effectively demonstrated the utility of fly traps for RHDV detection, the traps have never been adopted for monitoring purposes.

In this study we aimed to: i) assess the efficacy of fly traps as a virus monitoring tool with potential for use at landscape scales; ii) combine pooled fly samples with next-generation sequencing technology to measure the genetic variation in circulating RHDV at local scale; and iii) compare the RHDV variants observed in flies to those observed in local rabbits during two outbreaks. We present the successful use of wind-oriented fly traps for small-scale monitoring of RHDV strains in South Australia over a two-year period.

Methods

Field Sites

Five field sites in South Australia's Gawler/Barossa region were monitored during this study (Figure 1), each named for their locality. 'Rosedale 1' is a sheep grazing paddock containing a very dense population of rabbits sheltering in seven warrens and numerous piles of dense brush. 'Marananga' is a small vineyard with one known rabbit warren. 'Concordia' is a sheep

grazing paddock with one known, small and rather isolated, warren. 'Wasleys' is located on a farm near stockyards and barns, with no known rabbit warrens but evidence of rabbit activity. 'Rosedale 2' is a landholder's backyard with scattered roadside rabbit holes. 'Wasleys' and 'Rosedale 2' were added to the study part-way through 2013.

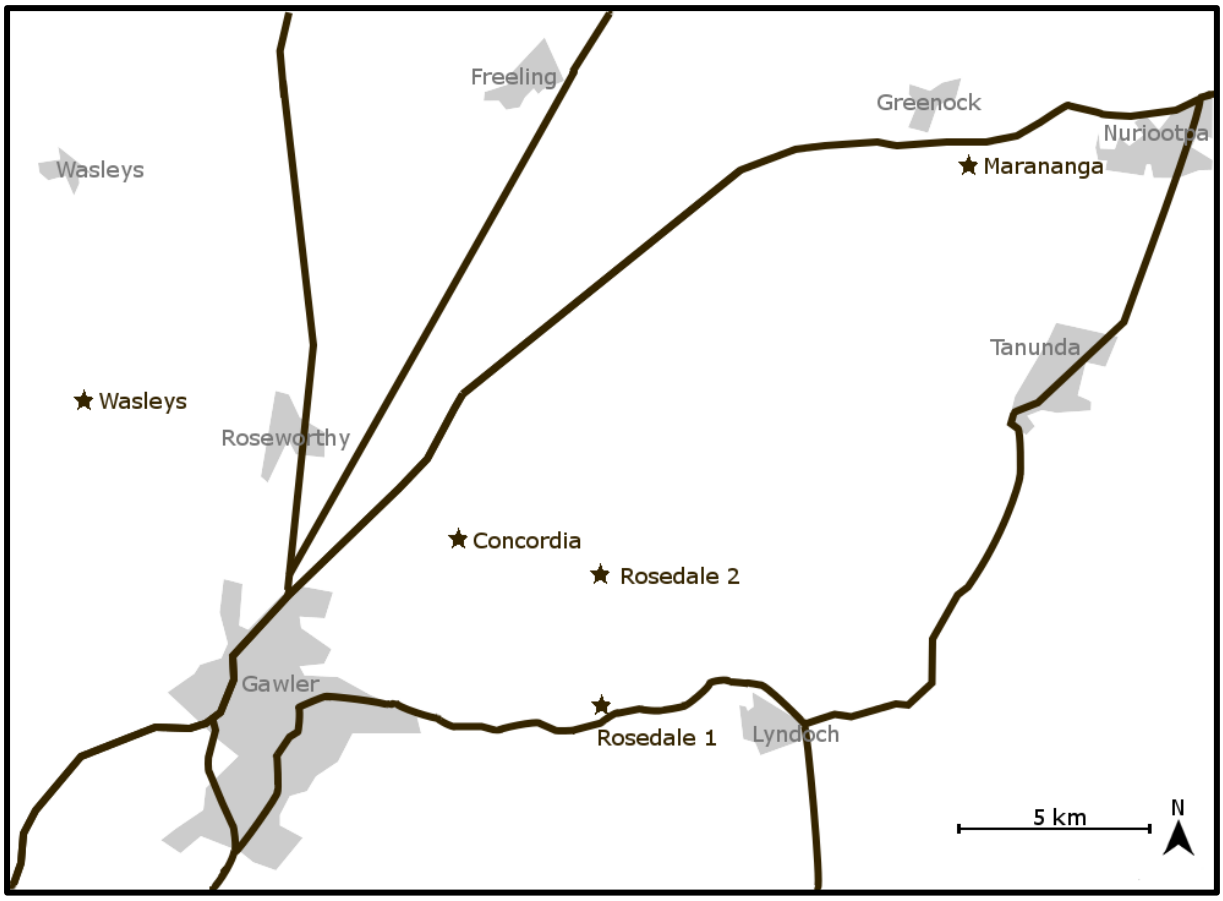

Figure 1: Site localities (black stars) for RHDV monitoring in the Gawler/Barossa region, South Australia, in spring 2013 and 2014. Townships are drawn in grey with major roads in black.

Sample Collection

Wind orienting fly traps (pictured in Figure 2) were constructed as described by Vogt *et al.* (1985) . One trap was established at each field site and baited with a slurry of minced liver, cattle or sheep dung, sodium sulphide (Na_2S) and water. The trap chamber where flies accumulated at each site was replaced approximately weekly throughout the expected spring RHDV1 outbreak season (August-November) and the bait rehydrated and topped up with liver. Trapped flies were identified by comparison with archived samples from the area, counted, and, with a swab of excreta from the sides of the trap chamber, frozen at -80°C until RNA extraction. Trap chambers were cleaned between uses with Pyroneg laboratory detergent to remove any previous traces of viral RNA.

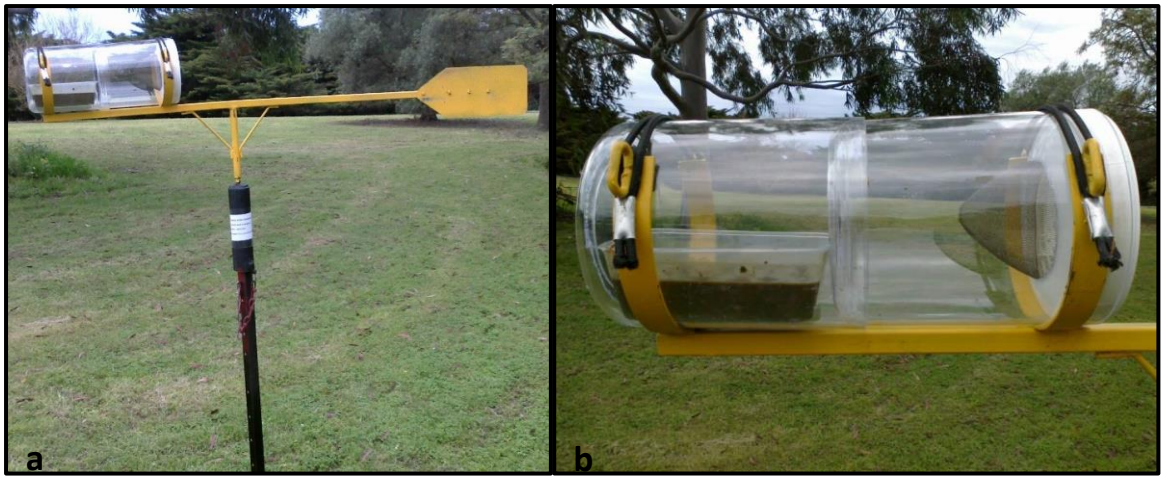

Figure 2: Wind orienting fly trap at Marananga. a) Full trap including base and wind-orienting vane. b) Close view of trap canister with bait in the left compartment and fly-accumulating compartment on the right where flies enter through a small hole in the tip of the gauze funnel.

On each visit every site was searched for rabbit carcasses including smelling and visual inspection of burrows using a torch. For each rabbit carcass recovered, necropsy was conducted on site and any visual signs of RHDV and timing of death were noted. These included the pale reticulated liver and dark enlarged spleen common in RHDV mortalities, as well as the presence and size of maggots for estimated date of death. Where carcasses were fresh, and not scavenged by foxes or birds, the liver was preferentially sampled (a known high source of virus (Ohlinger *et al.* 1990)), followed by other remaining organs. For older, significantly scavenged or decomposed carcasses, bone marrow was extracted from leg bones for viral RNA extraction.

Viral RNA extraction and sequencing

Up to a volume of 20ml of flies of mixed species were randomly subsampled from each trap and homogenised along with the swab of excreta in an approximately equal volume of TE buffer (10mM Tris, 1mM EDTA). RNA was extracted from 200ul of the resulting solution using the GeneJET Viral DNA & RNA Purification Kit, following manufacturer's instructions. RNA was extracted from rabbit tissue samples using the Qiagen RNeasy minikit following manufacturer's instructions. cDNA was amplified using the Invitrogen SuperScript® III First-Strand Synthesis System. RHDV VP60 capsid protein regions were amplified from the host-virus cDNA mixture by PCR using the primers RHDVf4846, RHDVr6059, RHDVf5926 and RHDVr6986 (Kovaliski *et al.* 2014), (primers detailed in Appendix 2, Supplementary Table 1) with New England Biosciences Q5® High-fidelity 2X Master Mix, using 40 PCR cycles with annealing temperature of 59°C. PCR products (a 1214 bp fragment beginning at position 4846 in the RHDV genome and a 1061 bp fragment beginning at position 5926) were visualised by agarose gel electrophoresis.

Amplicons were cleaned up using 0.5X Agencourt® Ampure beads, sheared using a Covaris instrument and libraries generated with Kapa DNA library preparation reagents for 150bp paired-end sequencing on an Illumina MiSeq at the ACRF Cancer Genomics Facility (Adelaide).

Sequence Filtering and Assembly

Primer regions were removed from read ends using the software Cutadapt (Martin 2011), discarding all reads of <35bp. Reads derived from rabbit carcasses were then aligned to RHDV reference genome NCBI NC_001543.1 using the Burrows-Wheeler Aligner BWA-MEM function. Geneious 9.1 (Kearse *et al.* 2012) was used to call a consensus sequence for each sample using the 'highest quality' setting, trimming any ends where coverage was less than 5x.

As we were seeking to test for the spatial and temporal presence of multiple RHDV variants in fly samples, a de-novo assembly approach was employed. Reads derived from fly pools were subset into replicate sets of 15k reads for each sample using a custom script. Using the Geneious implementation of BBTools v37.28 (Bushnell 2017), BBDuk was used to filter out read pairs with any read less than 100bp, BBMerge was used to merge paired reads with minimum overlap of 17 bases, Dedupe was used to remove duplicate reads with Kmer seed length 31bp and no substitutions permitted. BBNorm was then used to normalise reads to a target Kmer level of 50, minimum depth of 6. The resulting reads for each replicate set were de-novo assembled using MIRA 4.0 v1.1.1 (Chevreux *et al.* 2004) with accurate genome mode and no trimming. Resulting contigs were filtered to retain only those of >900bp length and consisting of at least 80 reads, for which consensuses were called using the Geneious 'highest quality' setting with minimum coverage of 6x. For each sample, contigs recovered from all replicates were pooled, deduplicted with Dedupe and then aligned to the reference sequence RHDV-V351 (GenBank accession KF594473.1). For contigs differing by <6 SNPs within each sample a single representative was retained in the alignment.

RHDV Variant Analysis

Two subsets of the RHDV alignment were selected for analysis. The first, which we refer to as the 2154bp alignment, comprised all contigs that were sequenced across the entire target 2154bp (N=67), allowing up to 100bp gap at the end of individual contigs. The second, which we refer to as the 1033bp alignment, comprised all contigs sequenced for the RHDVF5926/RHDVR6986 primer pair (N=131), with no more than 50bp end gap. Although this fragment is just 1033bp it contains the most variation and was sequenced in the most samples.

The software RDP4 (Martin *et al.* 2015) was used to screen for recombination events within each subset. This analysis used the RDP (Martin and Rybicki 2000), GENECOV (Padidam *et al.* 1999), MaxChi (Posada and Crandall 2001; Smith 1992), Bootscan (Martin *et al.* 2005) and SiScan (Gibbs *et al.* 2000) methods. Recombination events were accepted if supported by at

least three of the screening methods. Recombinant contigs were removed from the alignment prior to phylogenetic analysis.

Phylogenetic analysis was performed using RaXML as implemented in Geneious with GTR GAMMAI, rapid bootstrap+ML and 1000 bootstraps. RHDV sequence variants were assigned to clades based on phylogenetic output.

Data Analysis

The influence of site and date on total fly catch was investigated using negative binomial generalised linear models within the R software environment for statistical and graphical computing (version 3.2.5, R Core Team 2016). The R packages MASS v7.3-50 (Venables and Ripley 2002) and MuMIn v1.42.1 (Barto 2018) were used to average all subsets of the global model with fixed effects of site, date, number of trap days and year; with $\Delta AIC < 4.0$.

Overall RHDV detectability through carcass searches, and through fly traps, were compared using Mc Nemar's chi-squared tests in the R software package (version 3.2.5, R Core Team 2016).

The influence of site and date on RHDV variant detection was investigated using binomial generalised linear models in the R statistical environment (version 3.2.5, R Core Team 2016). The R packages MASS v7.3-50 (Venables and Ripley 2002) and MuMIn v1.42.1 (Barto 2018) were used to average all subsets of the global model with fixed effects of site, date and clade with deltaAIC of less than 4.

Results

Fly catch

Over 37,000 flies were caught during the monitoring periods, with an average of 605 per trap (range 14 - 4,255 flies, standard deviation 696.59).

51.8% of all flies caught were *Calliphora* spp. (*C. stygia*, *C. augur*, *C. dubia*), 28.0% were *Musca vetustissima*, and 7.0% *Chrysomya* spp. Species of *Hydrotaea* (3.3%), *Sarcophaga* (2.2%) and *Lucilia* (1.8%), were also present. Unidentified fly species comprised 4.7% of total catch and other insects 1.2%. Figure 3 illustrates the breakdown of catch by taxon for each site over time. *Calliphora* spp. dominated until October in both years, after which *Musca vetustissima* became prevalent in 2013.

Generalised linear modelling averaged results indicated that site and number of trap days were significant predictors of the total number of flies caught ($P < 0.0001$, see Supplementary Table 2 in Appendix 2 for model coefficients), but year and date within years were not. The sites 'Rosedale1' and 'Wasleys' caught significantly more flies on average and traps set for more days also tended to yield more flies.

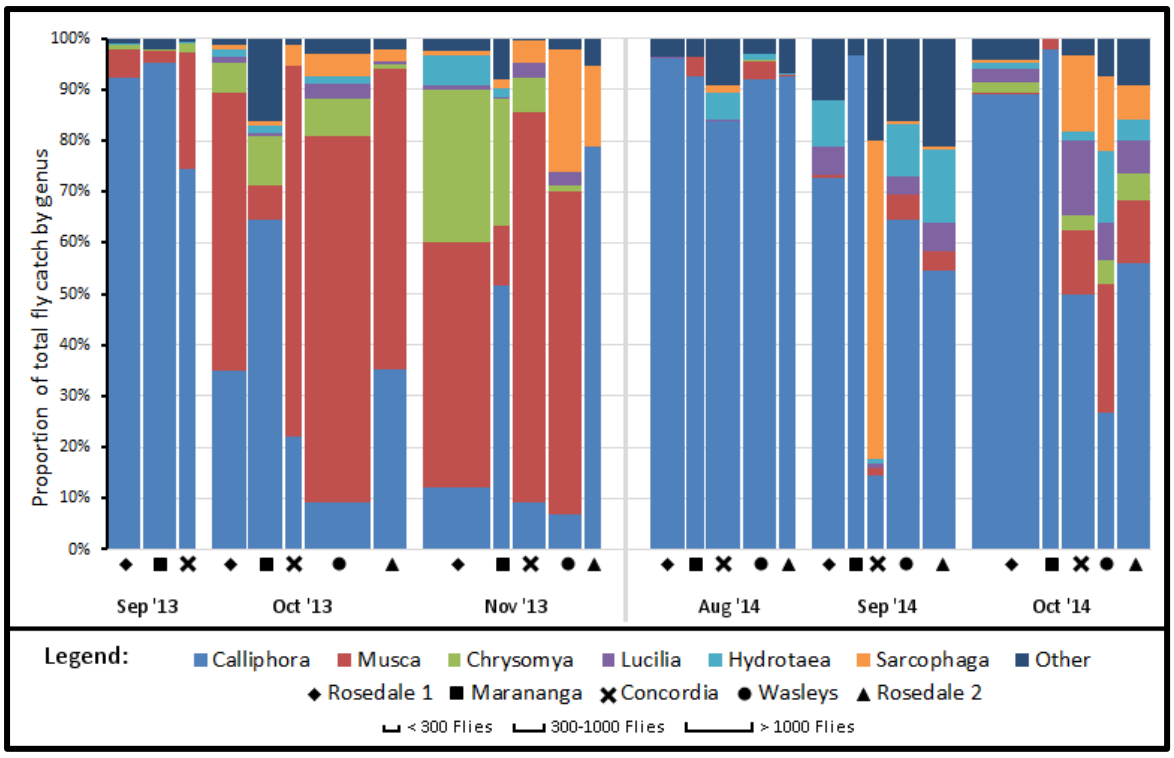

Figure 3: Monthly taxon distribution of flies trapped in the Gawler and Barossa regions, South Australia, around spring 2013 and 2014. Column widths are determined by total fly catch numbers divided by number of trap occasions for each month.

RHDV detection

Carcasses from rabbits that died of RHD were found at 'Rosedale 1' (n=13), 'Concordia' (n=7) and 'Rosedale 2' (n=3) in 2013, and 'Rosedale 1' (n=12) and 'Rosedale 2' (n=5) in 2014. Carcasses were found on a total of 17 out of 70 search occasions (24%). As all the rabbits at 'Concordia' seemingly died during the 2013 outbreak, evidenced by a lack of any fresh sign, and the warren had collapsed by early 2014, monitoring efforts during spring 2014 were reduced at this site.

We detected RHDV using PCR in 70% (49 out of 70) of fly samples, representing a significant increase in detection probability over carcass searches (24% (17 out of 70) detection in carcass searches; Mc Nemar's chi-squared test $\chi^2 = 30.118$, df = 1, $P < 0.001$). RHDV was detected on flies at all five sites (Figure 4), although not on every trip nor at all times when it would have been expected. For example, at 'Concordia' RHDV was not detected in a sample of flies collected on 10 October 2013 even though a rabbit killed by RHDV had been collected there two days earlier. Conversely, at 'Rosedale 1' in 2013 RHDV was detected on flies for a month before a dead RHDV positive rabbit was found (Figure 4). The carcass and fly PCR results (Figure 4) show that the timing of RHDV presence and outbreaks was approximately equal between sites, although in 2013 the outbreak at 'Concordia' occurred two weeks earlier than 'Rosedale 1' and 'Rosedale 2'. The virus was detected up to five

weeks prior to an outbreak ('Rosedale 1', 2013) and six weeks post outbreak ('Concordia', 2013).

Sequence variation

De-novo assembly of fly RHDV samples yielded up to 52 unique contigs of over 900bp per sample, with a mean of 7.9 contigs (median = 4 contigs, σ = 9.9). All samples with over 11 contigs were obtained from 'Rosedale 1'. There was no correlation between number of contigs in a sample and size of fly catch (r^2 = 0.0278) nor date (r^2 = 0.0114 in 2013, r^2 = 0.0059 in 2014). Contig counts are detailed in Appendix 2, Supplementary Table 3. 24 of 67 contigs in the 2154bp alignment and 46 of 131 contigs in the 1033bp alignment were identified as duplicates (i.e., identical to a contig from another site or date). RDP4 detected 16 recombination events affecting 51 contigs of 85 total in the 1033bp alignment, and 13 recombination events affecting 13 contigs of 43 total in the 2154bp alignment.

Phylogenetic analysis grouped the RHDV sequences into three clades (Figure 5). All contigs included in both the 2154bp and 1033bp alignments were assigned to the same clade by both analyses. The distribution of detected RHDV clades across time and space is presented in Figure 4. The clades do not show any spatial or temporal clustering within years at this regional scale. This stochasticity is supported by the generalised linear modelling results (Appendix 2, Supplementary Table 4) which recovered no significant influence of site, date or RHDV clade on detection in either year. Only Clades 1 and 3 were detected in rabbit carcasses. Clades 2 and 3 were frequently detected in both years while Clade 1 was only detected in 2014, when it was widely distributed across sites and dates. The sole exception was one 2013 carcass with an RHDV sequence identified as a duplicate of an RHDV sequence in a 2014 carcass assigned to Clade 1. On five occasions the clade present in a rabbit carcass was not detected in flies from the same sample site and date. All other carcass contigs had similar sequences present in the flies.

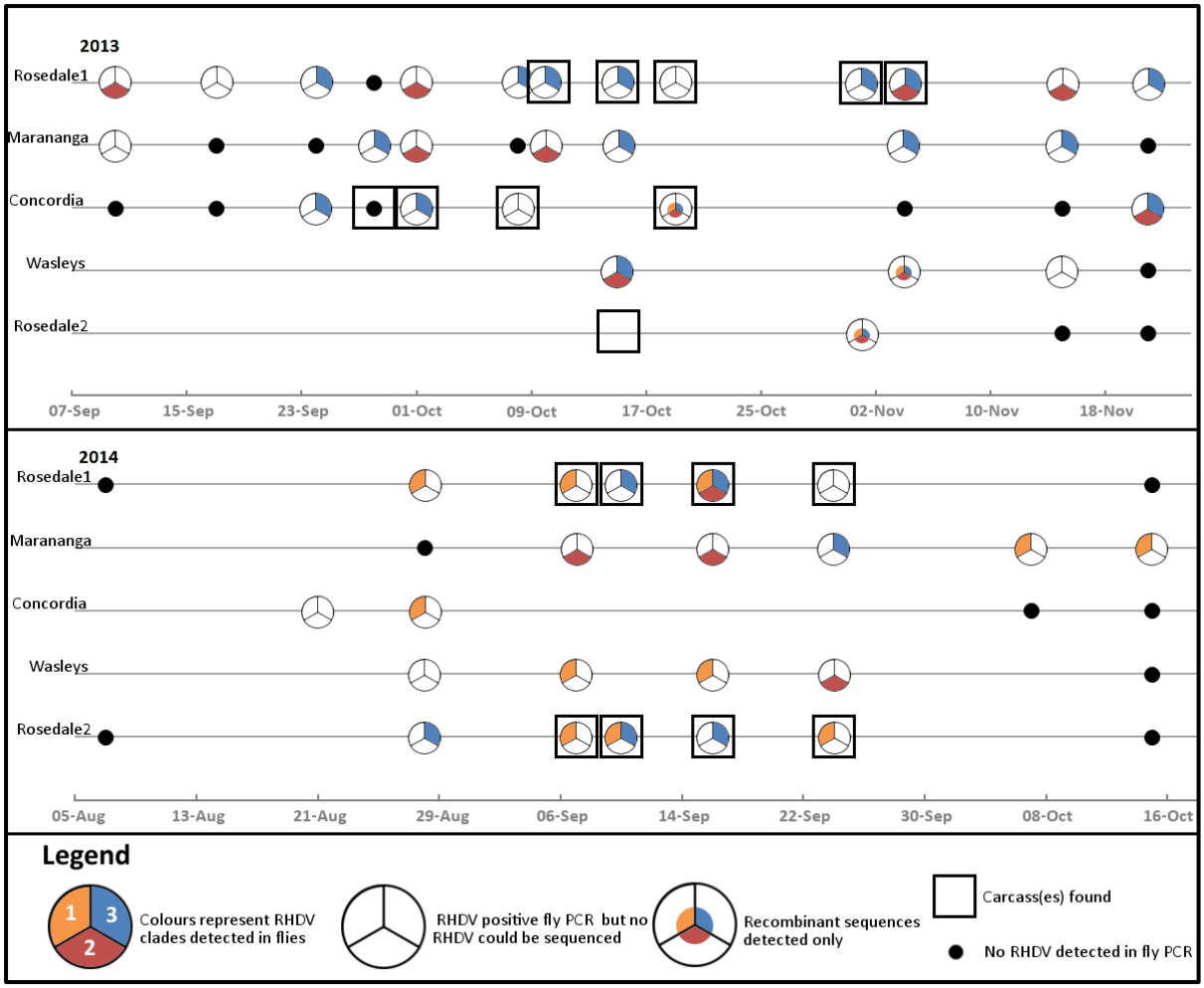

Figure 4: Timeline of RHDV presence in fly vectors over five field sites in the Gawler/Barossa region during the 2013 and 2014 outbreak seasons. Black squares indicate detection of at least one rabbit carcass with confirmed RHDV mortality. The three RHDV clades identified by RAxML are each represented by a pie chart segment; coloured segments indicate clade presence in flies, white segments indicate no detection of that clade. Black dots indicate negative RHDV PCR results for that fly sample. Note that the 2013 and 2014 x-axes do not align. No traps were set at Rosedale2 prior to the discovery of a carcass by the landholder on 15 Oct 2013.

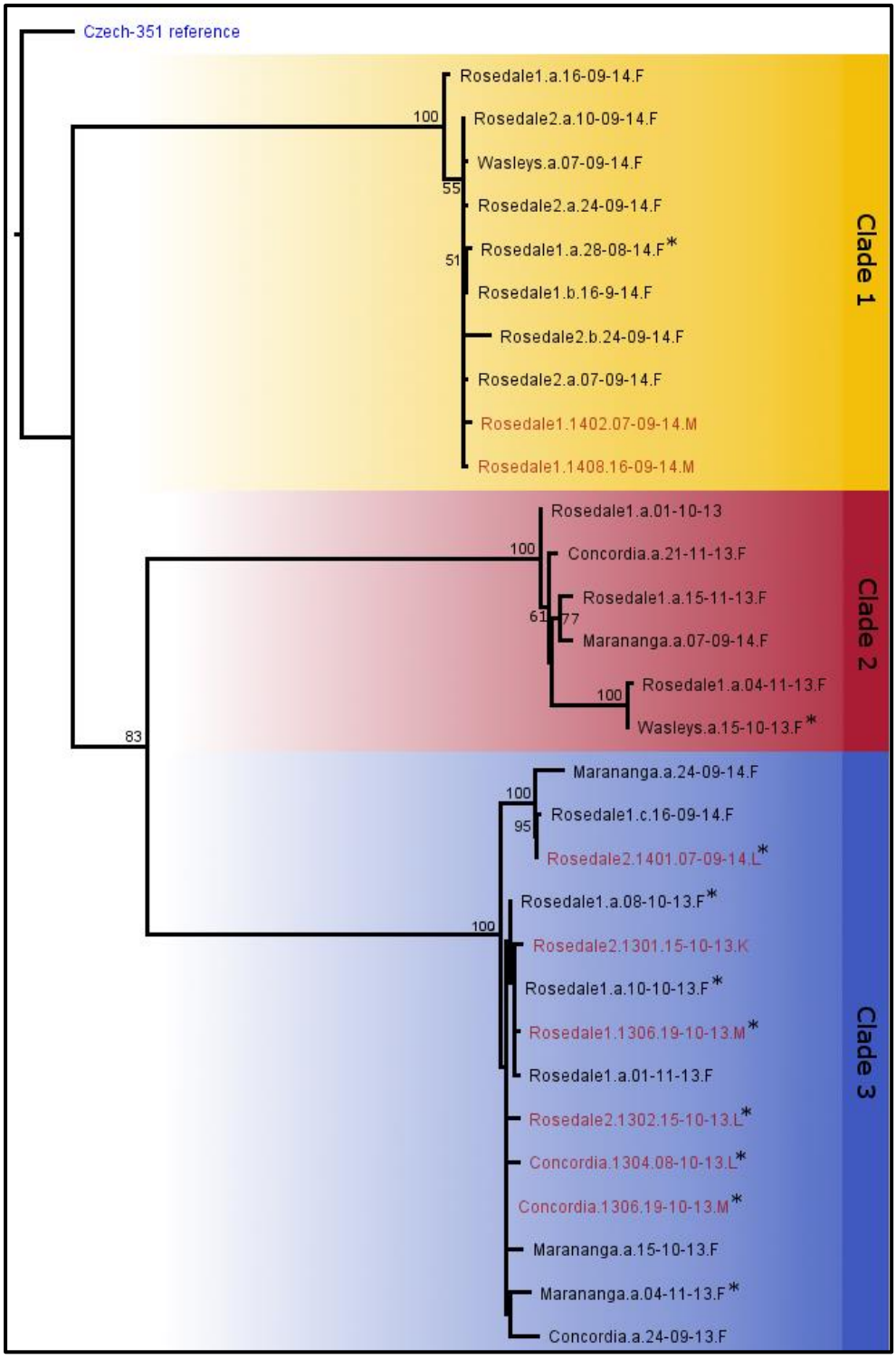

Figure 5: Maximum Likelihood phylogeny of 2154bp RHDV1 VP60 capsid sequences present in the Gawler/Barossa region during 2013 and 2014, rooted with the originally introduced Czech-351 strain from 1995 in blue. Node labels indicate bootstrap support values over 50. Isolates from fly samples are labelled in black, isolates from rabbit carcasses are labelled in red. Sample labels are in the format: Site.ID (numeric rabbit ID if carcass, sequence ID letter if flies).date(dd-mm-yy).organ (if carcass: L = liver, M = bone marrow, K = kidney, if flies: F). * indicates sequences with one or more duplicates found in other samples.

Discussion

Following the initial release of the Czech-351 strain of RHDV in Australia in 1995 the virus has evolved rapidly. Numerous variants have been recorded across Australia, primarily displaying high diversity in the VP60 capsid gene (Eden *et al.* 2015; Kovaliski *et al.* 2014). Schwensow *et al.* (2014) found that outbreaks of RHDV are most commonly initiated by strains that are circulating continuously between rabbit sub-populations. However, the geographic and temporal scale at which RHDV variation exists is poorly elucidated; a result of patchy, opportunistic sampling. This has contributed to a lack of clarity regarding the role of seasonal timing versus genetic changes in producing RHD outbreaks. Recently, the situation in Australia has been complicated by detection of two foreign strains, an RHDVa strain with genetic similarity to strains from China (Mahar *et al.* 2018b), and an RHDV2 strain resembling strains from Portugal and the Azores (Hall *et al.* 2015). While the RHDVa strain has not been reported beyond a limited area in New South Wales and the Australian Capital Territory, RHDV2 has spread rapidly across the nation and is largely replacing other circulating strains (Mahar *et al.* 2018a; Peacock *et al.* 2017); as previously occurred in Europe (e.g., Calvete *et al.* 2014; Le Gall-Reculé *et al.* 2013; Lopes *et al.* 2014). Furthermore, the intentional release of an RHDVa strain sourced from Korea, dubbed 'K5', occurred in 2017 (World Organisation of Animal Health 2017) in an effort to combat resistance to the Czech-351-derived field strain in rabbits previously exposed to benign RCV-A1 (Strive *et al.* 2013). Against this backdrop of competing viruses there have been calls for increased RHDV surveillance to assess the impact of new strains on existing epidemiological and rabbit demographic patterns.

Fly-traps for virus detection

The wind-oriented fly traps used in this study were effective for catching fly species known to carry RHDV in Australia, yielding up to 4,281 flies in a trap, although catch volume was affected by site and sampling variability. Traps set for longer durations showed an increase in total fly count on average, indicating that the bait remains at least somewhat attractive when unattended throughout a fortnight, even though we observed that the baits can become quite dry after a day or two in warm weather. Vogt *et al.* (1985) added blowfly larvae to their trap bait or stirred it hourly to increase bait attractiveness, which resulted in a higher fly yield. The additional effort and distasteful nature of these strategies are undesirable for widescale monitoring and citizen science projects. Therefore, to optimise future projects, we suggest that an experiment designed to estimate the duration of unstirred bait attractiveness is required.

RHDV1 was detected through onsite fly sampling up to six weeks before, as well as after, the discovery of rabbit carcasses (Figure 4). Furthermore, the virus was detected in flies from all sites including those at which no rabbit carcasses were ever discovered, despite extensive search effort. The comparatively low density of rabbits in populations where no carcasses were found suggests that the number of rabbit carcasses present (if any) was too low to be

detected. Rabbit populations at low density have higher proportional survival during outbreaks of RHDV (Henzell *et al.* 2002), limiting the pool of available carcasses. Additionally, because carcasses can be well camouflaged, located deep within warrens, or removed by scavengers, the number of detectable carcasses is substantially smaller than the total number of rabbit deaths. It is therefore unsurprising that carcasses are difficult to find where rabbit density is low. Our results reveal that detection of pathogens through their vectors is a powerful technique for the monitoring of fly-borne diseases, unhampered by the impact of low host population density on carcass availability, or carcass detection issues.

The apparent inability to detect RHDV1 in some fly samples, where virus might reasonably be expected, raises the possibility of false negative results. For example, at 'Concordia' a fresh rabbit carcass was found on 28th September 2013, but a negative result was obtained from flies collected on the same day. False negative results are likely to be obtained if a low proportion of flies at a site are carrying the virus and by chance none of these are trapped, or if the flies subsampled for RNA extraction by chance do not include those carrying the virus. Our methods mitigated this risk in two ways. First, the bait mixture following Vogt *et al.* (1985) is formulated for attractiveness to necrophagous flies, which are likely to have visited any rabbit carcasses in the area. The success of the bait specificity is demonstrated by the 94.1% of our total fly catch that were necrophagous species known to carry RHDV (Asgari *et al.* 1998; Barratt *et al.* 1998; McColl *et al.* 2002a), predominantly species of the genera *Calliphora* and *Musca*. The second measure was the inclusion of a swab of fly spots, which have been shown to carry RHDV (Asgari *et al.* 1998), from the trap walls. These fly spots may increase the pool of flies that can be sampled within a small volume as compared with whole fly bodies. While these two measures cannot guarantee that virus-bearing flies are sampled, the observed 70% positive PCR rate provided a much higher detection rate in this study than carcass-searches (24%). Inclusion of replicate fly traps in future monitoring projects will further reduce the chance of false negative results, as well as allowing detection probability to be calculated, providing an increased measure of confidence in virus presence/absence.

As well as improving detection rate over carcass-searches, vector-based monitoring was far more efficient. Continuous fly-trap monitoring required less than 10 minutes per site to collect and reset the trap, compared with up to two hours to search for rabbit carcasses at each of these sites. The fly trapping process required no prior experience or specific skill, unlike carcass searches which rely on the searcher's experience, observational skill and site familiarity. The simplicity of the fly trapping process lends itself to the use of citizen science for the establishment of extensive disease monitoring systems, both for RHDV and other fly-borne viruses. A community volunteer would be capable of operating a backyard fly trap and freezing the flies for transport to a processing laboratory with minimal effort. The fly traps themselves are cheap, reusable and durable – our traps remained completely serviceable after three years of continuous exposure to the elements.

Sequence variation in RHDV

The use of vector-based monitoring in this study gave us unprecedented insight into the extent of circulating RHDV variation. Next generation sequencing of fly-borne RHDV1 confirmed multiple co-circulating variants at a localised scale, facilitating frequent recombination events throughout an outbreak season. The importance of recombination in the evolution of RHDV has been postulated previously following the detection of recombination both within (Abrantes *et al.* 2008; Forrester *et al.* 2008; Kovaliski *et al.* 2014) and between (Lopes *et al.* 2014; Mahar *et al.* 2016; Mahar *et al.* 2018b) genotypes at national and international scales. The detection of 13-16 probable recombination events in this study evidences the frequency of recombination even among local variants, adding further weight to the importance of recombination for producing variation in this virus upon which selection can act.

Because contigs can vary by as little as one nucleotide, the three phylogenetic clades identified by RAxML present a more informative partitioning of RHDV variation than individual contigs in this study. We found significantly higher viral diversity at 'Rosedale 1' than the other study sites, which may be related to its high density of rabbit hosts, being the only site known to have multiple, large rabbit warrens.

The lack of spatial or temporal pattern observed in clade presence within years (the 'Clade', 'Site' and 'Date' terms were all non-significant in generalised linear modelling of RHDV1 detection) could reflect one of two scenarios: either the presence of RHDV variants at any given site is naturally stochastic, or the true pattern in regional cluster presence is obscured by false negatives. The latter option is supported by the apparent absence of outbreak-causing variants in some fly samples, even when present in a rabbit carcass from the same site and date.

All rabbit carcasses within a year at any given site had the same RHDV1 cluster present, suggesting that local outbreaks are caused by a single virus variant. This is not surprising given that infected rabbit carcasses can shed viable virus for at least 20 days (Henning *et al.* 2005; McColl *et al.* 2002b), making them a potent source of further infection in a population. It is therefore plausible that the first virus variant to achieve infection in a rabbit population with a sufficient density of susceptible rabbits (Wells *et al.* 2015) would achieve complete competitive dominance over other circulating strains in that population for that season. However, we did not detect a clear first variant to arrive in 2014 (Both Clade 1 and Clade 3 were first detected on August 28th), and in 2013 the first RHDV1 variant detected was from Clade 2 which, despite being present in both years, was the only clade not to be found in rabbit carcasses. While the absence of Clade 2 in carcasses could be an artefact of small sample size, the consistency of the clade found in carcasses within each site suggests that rabbit resistance to Clade 2 is more likely. That the same variants are successful in

seeding an outbreak at multiple sites implies the presence of a competitive advantage impacting strain infectivity.

Previous studies have highlighted the role of infectivity in RHDV evolution. Challenge studies found that resistance to RHDV1 in wild rabbit populations was associated with avoidance of productive infection (Elsworth *et al.* 2012), and that older RHDV1 variants with lower total mortality rates had a lower rate of infection as compared to recent field variants, while maintaining high mortality in infected individuals (Elsworth *et al.* 2014). Similarly, Mutze *et al.* (2014b) found a demographic shift in rabbit carcasses during RHDV1 outbreaks over 15 years, which suggested the virus had adapted to increase infectivity towards juvenile rabbits.

Recommendations for monitoring of fly-borne viruses

Our study effectively demonstrates that wind-oriented fly traps are ideal for use in wide-scale monitoring of fly-borne disease presence and evolution. They are a simple, sensitive and efficient monitoring tool. Manual carcass searches remain relevant for use during smaller scale intensive studies of outbreak epidemiology (such as Mutze *et al.* 2014b and Peacock *et al.* 2017) where demographic details of rabbit mortality are required. We recommend using multiple fly trap replicates at each monitoring site to decrease incidence of false negatives and enable calculation of pathogen detection probabilities. This method overcomes the limitations of carcass searching in low density host populations or where carcass loss or detection is problematic. It can be easily implemented by 'citizen scientists', and is ready for uptake in RHDV monitoring programs both in Australia and worldwide, with further potential for other fly-borne viruses such as avian influenza and Newcastle disease virus

Chapter 3 – Rabbit population structure

Statement of Authorship

Title of Paper	Genetic perspectives on the historical introduction of the European rabbit (*Oryctolagus cuniculus*) to Australia
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Iannella, A., Peacock, D., Cassey, P. and Schwensow, N., (2018) Genetic perspectives on the historical introduction of the European rabbit (Oryctolagus cuniculus) to Australia. *Biological Invasions*, pp.1-12. DOI: 10.1007/s10530-018-1849-2

Principal Author

Name of Principal Author (Candidate)	Amy Iannella		
Contribution to the Paper	Performed DNA extraction, amplification and reduced representation library preparation. Performed sequence data filtering, SNP identification and filtering and population structure analysis. Interpreted outputs, wrote manuscript and acted as corresponding author.		
Overall percentage (%)	90%		
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.		
Signature		Date	6 Nov 2018

Co-Author Contributions

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

iv. the candidate's stated contribution to the publication is accurate (as detailed above);

v. permission is granted for the candidate in include the publication in the thesis; and

vi. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	David Peacock		
Contribution to the Paper	Helped in data interpretation, manuscript evaluation and editing		
Signature		Date	18 December 2018

Name of Co-Author	Phillip Cassey		
Contribution to the Paper	Supervised development of work, helped to evaluate and edit the manuscript.		
Signature		Date	18-December-2018

Please cut and paste additional co-author panels here as required.

Name of Co-Author	Nina Schwensow		
Contribution to the Paper	Supervised development of work, helped in data interpretation, manuscript evaluation and editing		
Signature		Date	19/12/2018

The following is a post-peer-review, pre-copyedit version of an article published in *Biological Invasions*; edited for consistency and format of the thesis. The final authenticated version is available online at: https://rdcu.be/7mOv

Genetic perspectives on the historical introduction of the European rabbit (*Oryctolagus cuniculus*) to Australia

Abstract

The introduced European Rabbit (*Oryctolagus cuniculus*) is one of Australia's most damaging invasive alien species, both in terms of ecological and economic impact. Biological control of rabbits using the myxoma and rabbit haemorrhagic disease viruses has been undertaken in Australia since the mid-1950s, and locally varying genetic resistance to these biocontrol viruses has been reported. The efficacy of biocontrol agents may be influenced, among several factors, by the genetic background of rabbit populations. Therefore, understanding the invasion process of rabbits in Australia, and their resultant population structure, remains crucial for enhancing future rabbit management strategies. Using reduced-representation sequencing techniques we genotyped 18 Australian rabbit populations at 7617 SNP loci and show that Australia's invasive rabbits form three broad geographic clusters representing different ancestral lineages, along with a number of highly localised, strongly differentiated lineages. This molecular data supports a history of multiple independent rabbit introductions across the continent followed by regional dispersal, and the resulting patchwork genetic structure may contribute to variation across the country in rabbit resistance to the viral biocontrols. Our study highlights the importance of using genome-wide molecular information to better understand the historical establishment process of invasive species as this may ultimately influence genetic variability, disease resistance and the efficacy of biocontrol agents.

Introduction

The successful establishment of the European rabbit (*Oryctolagus cuniculus*) in Australia, and subsequent population boom after introduction by European settlers in the mid-1800s, is unsurprising given the species' generalist herbivorous diet and famously high fecundity (Tablado *et al.* 2009). However, the rapid spread of the rabbit throughout the southern half of Australia – described as the world's fastest mammal invasion (Caughley 1977) – contrasts starkly with modern understanding of rabbit ecology. Rabbits have small home ranges and are generally dependent on burrows for shelter against both predators and an inhospitable climate, making them poor natural dispersers. Studies indicate that dispersing sub-adult rabbits rarely travel further than 500m to a neighbouring pre-established warren, although rare dispersals over 1.5km have been recorded (Parer 1982; Richardson *et al.* 2002).

What then can explain the rapid colonization of the rabbit in Australia? Although a successful introduction of wild English rabbits by Thomas Austin of Barwon Park, Victoria in 1859 is commonly credited as the primary source of the original rabbit plague, evidence gathered from contemporary newspapers indicates that rabbit introductions were

commonplace in that period, with several additional documented successes (Peacock and Abbott 2013). This provides an alternative hypothesis: rather than dispersing continent-wide from a few primary introductions, rabbits were actively spread throughout the country by vagile human colonisers, dispersing under their own power at a more localised scale.

The historical dispersal pathways of the European rabbit in Australia are of modern consequence through their impact on the current genetic composition of rabbits, which in turn influences the effectiveness of pest management practices. Overgrazing by rabbits in Australia is responsible for an estimated AU$200 million in agricultural losses per annum (Cooke *et al.* 2013), as well as widespread damage to terrestrial ecosystems by preventing regeneration of palatable native plants (Bird *et al.* 2012; Cooke 2012; Mutze *et al.* 2016b) and supporting large populations of introduced predators (Holden and Mutze 2002; Pedler *et al.* 2016). Landscape scale management of rabbits has primarily been achieved through two introduced viral bio-controls: myxomatosis and rabbit haemorrhagic disease virus (RHDV). Geographic differences in biocontrol efficacy have been observed. By challenging rabbits from populations across the country with low doses of RHDV, Elsworth *et al.* (2012) found evidence of varying resistance to the virus across populations. This may be due to varying selection pressures acting on the locally available genotypes of individual rabbits (Schwensow *et al.* 2017a; Schwensow *et al.* 2017b).

To investigate the genetic differentiation among invasive Australian rabbit populations, we used double-digest Genotyping-by-sequencing (ddGBS, (Poland *et al.* 2012)) to detect genome-wide SNPs in N = 413 rabbits sampled continent-wide across 18 populations. As well as the inherent improved insights into the colonisation of Australia by the rabbit, understanding the genetic structure of Australian invasive rabbits will have two demonstrable benefits for pest management strategies. Firstly, the extent of genetic variation between rabbit populations may reflect varied potential for developing resistance to RHDV, myxomatosis, or any future new biocontrols. Documenting this variance will thus guide understanding of the variation that can be expected in efficacy of present and future nationwide biocontrol initiatives. Secondly, the degree of connectivity between rabbit populations will influence the size of effective rabbit management units such that re-immigration from neighbouring areas following control activities is minimised, as has previously been implemented for rats on South Georgia (Robertson and Gemmell 2004) and feral pigs in Australian rangelands (Cowled *et al.* 2008).

Methods

Sample collection

Ear tissue was scavenged from wild rabbits shot throughout 2014; during regular activities of readers of the Australian Shooter magazine. Tissue was immediately stored in DESS (20% dimethyl sulphide, 0.25M disodium EDTA, saturated with NaCl), and frozen at -20°C upon receipt. The GPS location of each sample was recorded, with 18-49 rabbits sampled from

within a maximum 65km radius at each of 11 sites throughout Western Australia (WA), South Australia (SA), Victoria (Vic), Australian Capital Territory (ACT) and New South Wales (NSW). Genomic DNA was also obtained from rabbits trapped in 2009 at eight additional sites, including Southern Queensland (QLD) during a previous challenge study experiment by Elsworth *et al.* (2012) and from a site at Turretfield in SA collected in 2014 as part of an ongoing capture-mark-recapture study. All sample locations are summarised in Table 1; see Results section for map.

Table 1: Rabbit sample locations used in this study, grouped by state. N reflects final sample numbers after sequence filtering (total N= 413). Population cluster as determined by majority ancestry in fastSTRUCTURE. State abbreviations: WA - Western Australia, SA - South Australia, QLD - Queensland, NSW - New South Wales, ACT - Australian Capital Territory, VIC - Victoria.

SITE	STATE	POPULATION CLUSTER	MEAN LONGITUDE	MEAN LATITUDE	N	COLLECTION YEAR
WA1	WA	Perth	114.2034	-27.8897	17	2014
WA2	WA	Perth	115.9746	-29.9885	18	2014
WA3	WA	Perth	117.8554	-34.7799	47	2014
SA1	SA	Perth	135.0620	-33.5638	15	2014
SA2	SA	Central	137.9865	-27.8148	17	2014
SA3	SA	Central	138.6361	-31.4547	18	2009
SA4	SA	Adelaide	138.8222	-34.5604	15, 20	2009, 2014
QLD	QLD	Central	142.5956	-25.9008	10	2009
NSW1	NSW	Central	144.9076	-35.5975	34	2014
NSW2	NSW	Central	149.4667	-33.3600	12	2009
NSW3	NSW	Sydney	150.7676	-34.0721	19	2014
NSW4	NSW	Central	151.8194	-29.3097	17	2014
NSW5	NSW	Brisbane	152.0294	-29.0286	20	2014
ACT	ACT	Central	149.3934	-35.0893	37	2014
VIC1	VIC	Central	142.4164	-34.6342	10	2009
VIC2	VIC	Melbourne	142.0439	-38.3308	11	2009
VIC3	VIC	Melbourne	144.1424	-37.5883	22, 38	2009, 2014
VIC4	VIC	Melbourne	144.3736	-37.0175	16	2009

DNA extraction and sequencing

DNA was extracted from roughly 6mm^2 of each rabbit ear tissue sample using the Gentra Puregene tissue extraction protocol after desalting in 1ml TE buffer for at least 1 hour, and eluted in 100µl TLE buffer. Approximately 200ng of whole genome DNA extract was used to prepare a double digest GBS library following the protocol of Poland *et al.* (2012), using New England BioLabs PstI-HF as enzyme 1 and New England BioLabs MspI-HF as enzyme 2. GBS

library was purified using the Qiagen QIAquick PCR Purification Kit, and fragments <200bp in length removed using 1.1x ratio of Agencourt AMPure XP beads. Ninety-six samples including a negative control were pooled per run for 75 bp single-end sequencing by the Australian Genome Research Facility on an Illumina NextSeq 500.

Genotyping and SNP filtering

Raw sequence reads were filtered for quality (sliding window phred score limit of 10) and adapter presence, trimmed to 40bp and demultiplexed using the process_radtags program from the software Stacks v1.34 (Catchen *et al.* 2013). Reads were then mapped to the rabbit genome assembly OryCun2.0 (available from the National Center for Biotechnology Information (NCBI) at www.ncbi.nlm.nih.gov/genome/316?genome_assembly_id=203429) using the bwa aln/samse functions from the software Burrows-Wheeler Aligner (Li and Durbin 2009) with default values. The Stacks v1.34 pipeline ref_map was then used to call SNPs with a minimum stack depth of 10, minimum minor allele frequency of 0.01 and minimum of 80% of samples represented. Files were checked for quality at each stage using FastQC. Based on the Stacks sumstats output, loci that mapped to the X-chromosome (NC_013690.1) were removed from the dataset along with one member of any pair of loci that mapped within 20,000 bp of each other to minimise linkage between loci. Rabbits with >40% missing data were removed from the dataset.

Loci under selection can potentially bias analysis of population structure (Beaumont and Nichols 1996), therefore loci with outlying F_{ST} values were identified for removal using the software package Bayescan 2.1 (Foll and Gaggiotti 2008). Bayescan 2.1 was run with prior odds of 100 for the neutral model, sample size of 5,000, a thinning interval of 10, twenty pilot runs of length 5,000 and a burn-in of length 50,000. Samples were grouped into 'populations' based on sample site. Any locus detected as an F_{ST}-outlier at a false discovery rate of 0.05 was removed from the dataset.

Analysis of population structure

Population structuring and sample ancestry estimation was investigated using three approaches: (i) multivariate analysis through discriminant analysis of principal components (DAPC) with the R package adegenet 2.0.1 (Jombart *et al.* 2010); (ii) population genetic model-based Bayesian clustering with the program fastSTRUCTURE (Raj *et al.* 2014); and (iii) a spatially explicit least-squares optimisation approach with the package Tess3R (Caye *et al.* 2016) run through R 3.2.5 with Rstudio 1.0.136. Tess3R differs from fastSTRUCTURE in its inclusion of geographic proximity information and a model-free algorithm, whereas fastSTRUCTURE is based on population genetic models of Hardy-Weinberg equilibrium.

Genetic clusters for DAPC were inferred through K-means clustering with the find.clusters function. The optimal value for K was chosen at the minimum Bayesian information criterion (BIC) score using 20 replicates of $1x10^7$ iterations for K=1-25. DAPC was then used for each sample to assign membership probabilities to these clusters. To avoid overfitting, the

number of principal components retained during DAPC was selected using the cross-validation function. fastSTRUCTURE was run for K=1:12 with a simple prior. Tess3R was run with ten replicates for each of K=1:20 under default parameters. Admixture proportions for the four most supported values of K for fastSTRUCTURE and Tess3R were visualised through the barplot.tess3q function of Tess3R. Where several sample sites were supported to form an ancestral cluster those site clusters were re-run separately in fastSTRUCTURE, using both the simple and logistic prior, in order to examine substructure within the cluster.

GenAlEx v6.503 (Peakall and Smouse 2012) was used to find overall F_{ST} value through Analysis of Molecular Variance (AMOVA) with 9,999 permutations and to perform Mantel tests for isolation-by-distance through correlation between linear genetic distance and both linear and log-linear geographic distance with 9,999 permutations. Expected and observed heterozygosity, average number of alleles per locus and number of private alleles were also calculated for fastSTRUCTURE clusters normalised to 19 individuals each (equivalent to the total sample size of the smallest cluster). Arlequin 3.5.2.2 (Excoffier and Lischer 2010) was used to generate a pairwise F_{ST} matrix, F_{ST} significance was assessed with 10100 permutations using α=0.05 with Bonferroni correction.

Results

Sequencing recovered 1,675 million reads yielding 39,753 SNP loci with over 10x coverage, of which 7,821 remained after quality control and filtering. At a false discovery rate of 0.05 Bayescan identified 204 outlier loci, yielding a set of 7,617 selectively neutral loci for population structure analysis, typed across 413 rabbits from 18 sampling locations.

k-means clustering supported an optimum of five clusters to best describe the rabbit population structure of our continent-wide rabbit samples, with the Bayesian Information Criterion forming a distinct minimum ‘elbow’ at this point for 9 of 20 replicates and a further 5 replicates ambiguous between k=4 and k=6. Cross-validation supported retention of 50 principal components during DAPC. Three of these clusters (dubbed Central, Melbourne and Perth) represent contiguous geographic regions, while the remaining clusters represent individual sample sites SA4 and NSW3. DAPC based on the five clusters detected minimal overlap between clusters (Figure 1). The first discriminant function clearly separated clusters Adelaide and Perth from the remaining groups, while the second isolated Sydney, and a third discriminant function clearly distinguished all clusters.

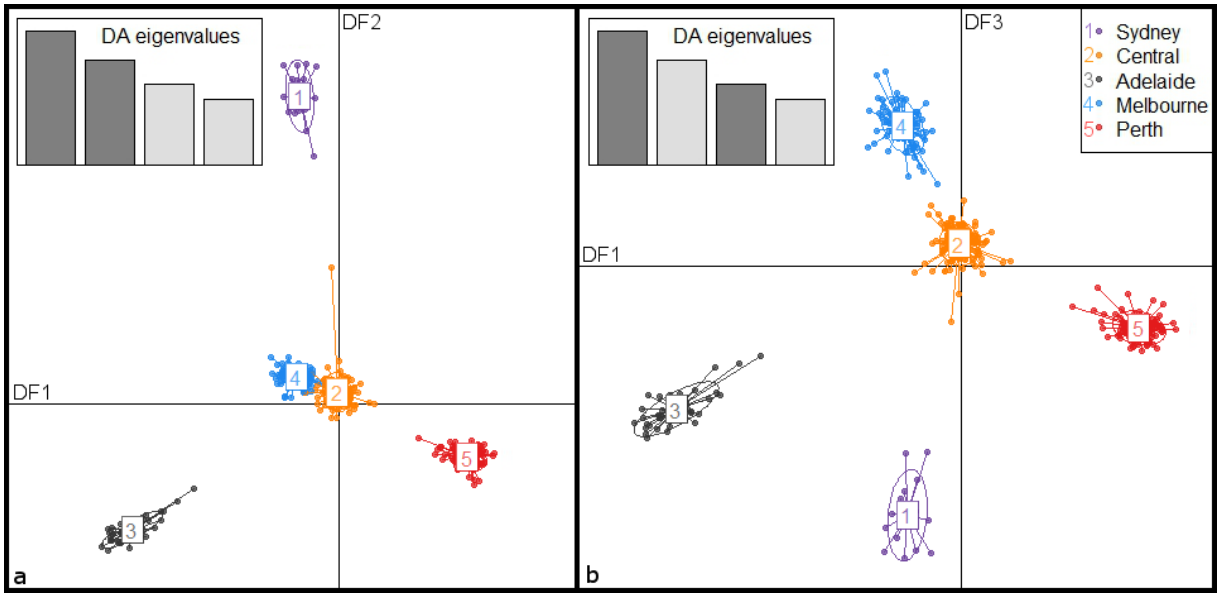

Figure 1: Discriminant Analysis of Principal Components (DAPC) scatter plot of five Australian rabbit clusters, produced using the adegenet package of R. Insets show relative eigenvalues for the four discriminant functions (plotted functions in darker grey). a) X-axis represents discriminant function 1, y-axis represents discriminant function 2. b) X-axis represents discriminant function 1, y-axis represents discriminant function 3

The chooseK component of FastSTRUCTURE analysis supported six population clusters at the maximum marginal likelihood value; while Tess3R did not support a distinctive best value for K, suggesting that further sub-structuring is present within the six clusters. Independent population clustering by both fastSTRUCTURE and Tess3R (Figure 2) showed groupings largely congruent with those supported by k-means clustering in adegenet. All sites from Western Australia along with SA1 in South Australia formed a consistent group (predominantly red in Figure 2, cluster hereafter called Perth), while sites in southern Victoria formed a second consistent grouping (predominantly blue in Figure 2, cluster hereafter called Melbourne). Sites SA4, NSW3 and NSW5 each represented distinct genetic lineages, hereafter named for their nearby state capital cities Adelaide, Sydney and Brisbane (grey, purple and green respectively in Figure 2), while the remaining central and eastern sites formed a large cluster with varying levels of admixture from neighbouring clusters (predominantly yellow sites in Figure 2, which we will hereafter refer to as the Central cluster). Of the 413 samples, 379 were assigned to the same groups by both fastSTRUCTURE and Tess3R, although Tess3R generally supported a greater level of admixture compared to fastSTRUCTURE (Figure 2b). Of the 34 samples

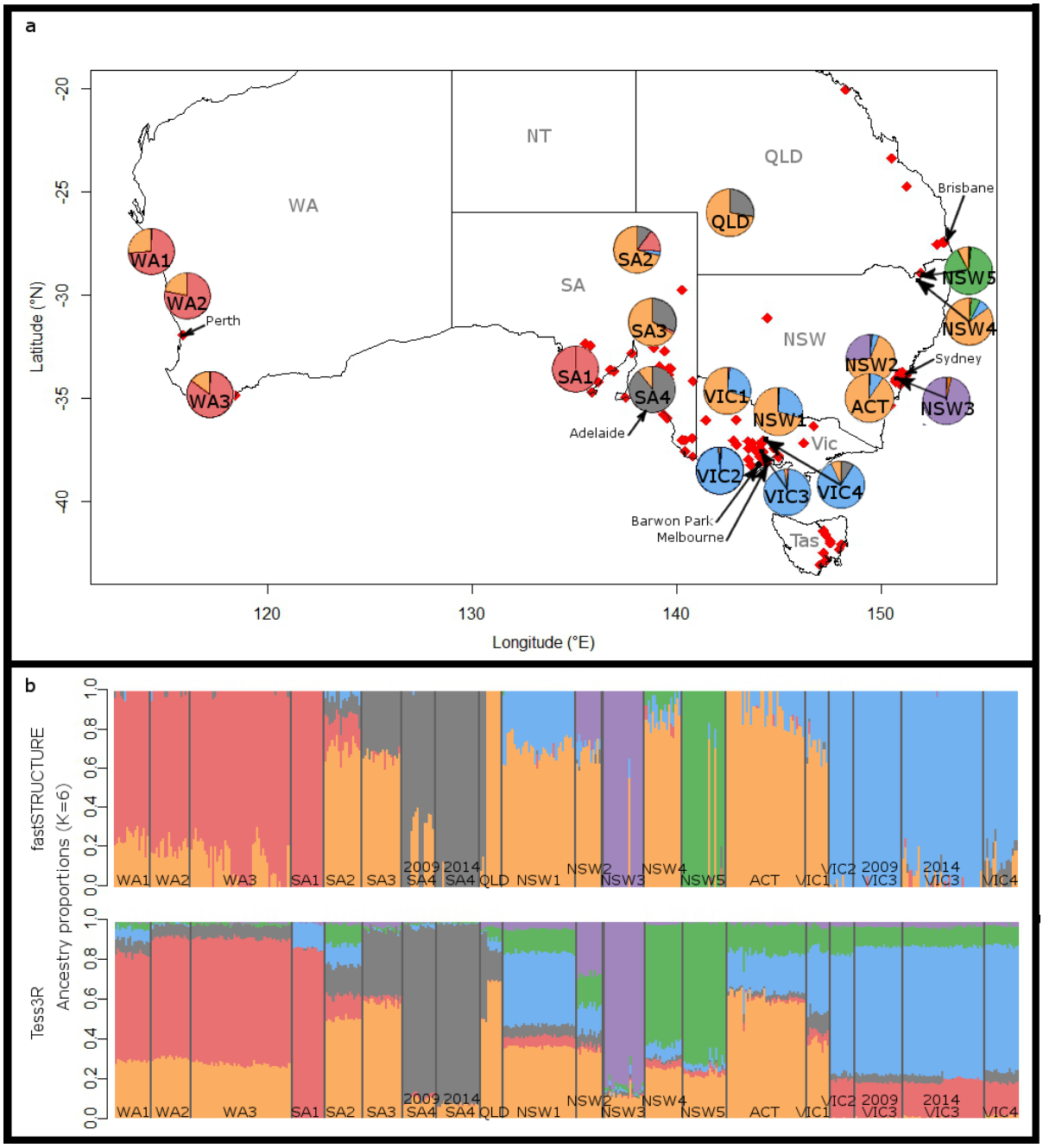

Figure 2: (a) Map of rabbit sample sites. Pie chart colours represent proportions of ancestry for rabbit sample sites used in this study as estimated by fastSTRUCTURE with K=6. Sample sites numbered from west to east in each state, as in Table 1. Historical rabbit introduction records of successful or unknown outcome reported by Peacock and Abbott (2013) are represented as red diamonds, noting some may be hidden by pie charts. The Barwon Park release site in Victoria and state capital cities are specifically indicated. (b) Bar charts of individual rabbit ancestry proportions as estimated with K=6 by fastSTRUCTURE (above) and Tess3R (below). Individuals grouped by sample site, numbered as in a). Figure produced using GNU Image Manipulation Program and the R packages Tess3R, maps, mapplots and oz.

which differed between the two programs 17 were from Site NSW4 which was assigned to the central cluster by fastSTRUCTURE but grouped with the neighbouring Brisbane cluster by Tess3R. When examined separately in both fastSTRUCTURE and Tess3R each of the three broader geographic clusters showed evidence of further substructuring aligning strongly with sample site location.

Pairwise F_{ST} values between sampling locations ranged from 0.007 - 0.247 and only one of 190 site pairs was not significantly different (see Figure 3). In line with population clustering results F_{ST} values were generally larger for comparisons involving SA4, NSW3 or NSW5. SA1 also had high pairwise F_{ST} values with all pairs outside of Western Australia (mean F_{ST} = 0.151 , σ = 0.039), particularly when paired with neighbouring SA4 (F_{ST}=0.247). Pairwise F_{ST} values between years at SA4 and at VIC3 were low (0.055 and 0.021 respectively) but still significant.

	WA1	WA2	WA3	SA1	SA2	SA3	SA4 (2009)	SA4 (2014)	QLD	NSW1	NSW2	NSW3	NSW4	NSW5	ACT	VIC1	VIC2	VIC3 (2009)	VIC3 (2014)	VIC4
WA1	0																			
WA2	0.017	0																		
WA3	0.018	0.029	0																	
SA1	0.069	0.080	0.075	0																
SA2	0.029	0.048	0.046	0.114	0															
SA3	0.060	0.078	0.072	0.159	0.021	0														
SA4 (2009)	0.102	0.114	0.105	0.194	0.065	0.076	0													
SA4 (2014)	0.161	0.173	0.164	0.247	0.126	0.115	0.055	0												
QLD	0.073	0.088	0.081	0.167	0.035	0.083	0.100	0.143	0											
NSW1	0.050	0.066	0.060	0.122	0.027	0.052	0.087	0.150	0.054	0										
NSW2	0.059	0.076	0.067	0.143	0.032	0.089	0.131	0.169	0.120	0.038	0									
NSW3	0.141	0.152	0.139	0.195	0.112	0.137	0.166	0.221	0.142	0.115	0.086	0								
NSW4	0.059	0.071	0.063	0.137	0.026	0.066	0.105	0.163	0.068	0.038	0.045	0.129	0							
NSW5	0.109	0.126	0.116	0.191	0.085	0.117	0.156	0.212	0.115	0.090	0.095	0.175	0.079	0						
ACT	0.047	0.067	0.062	0.126	0.028	0.055	0.091	0.151	0.055	0.031	0.038	0.122	0.029	0.084	0					
VIC1	0.069	0.088	0.077	0.153	0.040	0.089	0.137	0.178	0.099	0.045	0.086	0.140	0.062	0.114	0.047	0				
VIC2	0.054	0.071	0.057	0.117	0.037	0.117	0.156	0.187	0.145	0.026	0.123	0.115	0.055	0.104	0.035	0.094	0			
VIC3 (2009)	0.054	0.075	0.063	0.120	0.045	0.111	0.146	0.187	0.126	0.040	0.105	0.123	0.062	0.112	0.048	0.092	0.088	0		
VIC3 (2014)	0.049	0.068	0.064	0.102	0.046	0.077	0.110	0.171	0.074	0.038	0.046	0.110	0.051	0.106	0.049	0.051	0.007	0.021	0	
VIC4	0.065	0.085	0.080	0.136	0.054	0.085	0.115	0.171	0.099	0.050	0.069	0.128	0.068	0.121	0.060	0.072	0.057	0.062	0.050	0

Figure 3: Pairwise site F_{ST} matrix, as determined by Arlequin 3.5.2.2 (Excoffier and Lischer 2010). Colour gradient with green = lower differentiation between populations, red = higher differentiation between populations. All values significant at α = 0.05 with bonferroni correction except SA2/NSW2.

AMOVA testing found significant differentiation in the sample sites (F_{ST} = 0.108, *P* <0.001) with 7% of molecular variance apportioned to differences among sites. A further 3% of molecular variance was attributed to differences among clusters as identified by fastSTRUCTURE, and 23% to differences among individuals. We did not find evidence of correlation between genetic and geographic distance (Mantel test simulated *P* = 0.293 and 0.482 for geographic distance and log(1+geographic distance) respectively).

The total number of private alleles for each cluster (prior to normalisation) were: Perth = 4, Central = 20, Melbourne = 11, Adelaide = 2, Sydney = 15 and Brisbane = 2. Private alleles in the larger clusters were, on average, present at lower frequency than those in clusters

comprising a single sampling site (mean private allele frequency 7.5 % vs 20.9 %, *P* <0.0001). Clusters that comprised multiple sampling sites had higher genetic diversity than clusters that comprised a single sampling location, shown by greater allelic richness (mean number of alleles per locus) (1.846 - 1.905 vs 1.731 - 1.803) and greater expected heterozygosity (26.8-28.6 % vs 23.0 - 26.3 %). Cluster genetic diversity is detailed in Table 2.

Table 2: Genetic diversity statistics by population cluster. N=19 randomly chosen individuals per cluster.

	CENTRAL	PERTH	MELBOURNE	ADELAIDE	SYDNEY	BRISBANE
MEAN ALLELIC RICHNESS	1.905	1.846	1.894	1.732	1.803	1.731
PRIVATE ALLELES	53	35	89	22	84	10
MEAN EXPECTED HETEROZYGOSITY	0.283	0.268	0.286	0.230	0.263	0.241
MEAN OBSERVED HETEROZYGOSITY	0.271	0.246	0.272	0.225	0.251	0.254

Discussion

A history of multiple introductions

Caughly's (1977) observation of Australia's colonisation by rabbits as being the fastest mammal colonisation in history is oft-cited and fits well with the social narrative of Australia's overwhelming rabbit plagues. While the rapid initial spread of rabbits across this landscape is undisputed, the popular notion that rabbits achieved this feat through natural dispersal in a single wave from Barwon Park has been challenged by historical evidence of multiple introductions by Stodart and Parer (1988) and Peacock and Abbott (2013). The molecular evidence presented here supports the historical records, in suggesting that while natural dispersal has likely played a large role in regional range expansions, continent-wide movement was also facilitated by repeated human-mediated introductions.

Analyses of Australia's rabbit population structure through three independent approaches based on principal components (DAPC), bayesian clustering (fastSTRUCTURE) and spatially explicit least-squares optimisation (Tess3R) all yield a concordant notion of six genetic lineages. Of these, three are geographically broad lineages roughly representing Western Australia (Perth), Southern Victoria (Melbourne) and Central Australia/New South Wales (Central), with evidence of moderate admixture across their boundaries (as in Figure 2). The remaining three comprise individual sample sites - SA4 (Adelaide), NSW3 (Sydney) and

NSW5 (Brisbane) - and are notable for their much greater genetic differentiation at a very localised geographic scale.

Remarkably, fastSTRUCTURE suggested very little common ancestry between rabbits from NSW4 and NSW5, despite the two sites being separated by less than 40 km and with no readily apparent geographic or biological barrier. Tess3R's grouping of NSW4 and NSW5 is the most substantial departure from the fastSTRUCTURE results, and is likely a result of the spatial constraints in the Tess3 algorithm which ensure that geographically close populations are more likely to share ancestry than those that are far apart (Caye *et al.* 2016). It appears that NSW4 and NSW5 lie at the boundary of the Central and Brisbane ancestral groups, and represent admixed populations. Substantial admixture is indicated both in the uncertain ancestry of NSW4 (Figure 2) and the unexpectedly high heterozygosity present in NSW5 when compared to other clusters (Table 2).

The substantial differentiation of rabbits in the Greater Western Sydney area (NSW3) is further supported by moderately high pairwise F_{ST} values with all other sites (0.086-0.221) and the presence of 84 private alleles (many at high frequencies), confirming the results of Phillips *et al.* (2002) who tentatively found differentiation between rabbits in Sydney and surrounding areas using allozyme data.

The strong structuring with admixture along cluster boundaries observed in Australia's rabbit population is consistent with two possible scenarios, genetic drift or multiple introductions, both of which are not mutually exclusive. A single introduced population experiencing a dramatic decrease in migration following nation-wide dispersal could have formed pockets of differentiation as a result of genetic drift. Rabbits in Australia have undergone repeated population bottlenecks driven by control activities. At introduction in 1950 myxomatosis caused up to 99% rabbit mortality (Fenner *et al.* 1953), and initial RHDV mortality was recorded as high as 95% in 1995 (Mutze *et al.* 1998). Bottlenecks such as these compound the effect of genetic drift in isolated populations because rare alleles present in surviving individuals become disproportionately common among their descendants, while lost alleles are not quickly replaced by mutation or immigration. In this manner more isolated populations such as those at sites SA4, NSW3 and NSW5 may have become differentiated from surrounding sites. There is some limited evidence for a change in allele frequencies over time at our SA4 site where the pairwise F_{ST} between samples taken in 2009 and 2014 was low (0.055) but still significant, suggestive of ongoing genetic drift.

The observed population structure may also result from multiple independent introductions to different regions which are experiencing admixture at the boundaries of dispersal. These independent historical introductions can account for the genetic differentiation of population clusters through either differing rabbit source populations or founder effects caused by strong population bottlenecks in isolated populations at introduction. A

combination of genetic drift and multiple introductions is also possible. It may be that some ancestral clusters result from isolated populations experiencing strong genetic drift, while others represent unique introduction events. Without comparative genetic information from source populations it is difficult to distinguish between these alternative histories, however, two factors lead us to suggest that multiple independent introductions are likely to have contributed to the differentiation between our genetic clusters. Isolation-by-distance was not observed at a continent-wide scale, which would have been expected in a range expansion from a single successful introduction such as from Barwon Park. Although genetic drift could reduce the correlation between genetic and geographic distance over time, the broad scale of clusters and extent of admixture observed at cluster boundaries suggests that the clusters may not be sufficiently isolated for drift to cause such strong differentiation. Further, the presence of numerous private alleles in each population cluster can more parsimoniously be explained by multiple introductions than by mutation in just 150 years. The particularly high number of private alleles in the Melbourne and Sydney clusters in particular suggest that several introductions may have contributed to the gene pools in these locations. Multiple introductions is supported by contemporary accounts (Peacock and Abbott 2013).

Our support for multiple historic rabbit introductions in Australia challenges the conclusions of Zenger *et al.* (2003) who investigated the genotypic variation of Australian rabbits by comparing seven microsatellite markers in five Australian rabbit populations to those from European sourced rabbits. Zenger *et al.* (2003) interpreted their microsatellite diversity within the context of isolation-by-distance, assuming that all rabbit populations in Australia stemmed from the Barwon Park plague via a series of range expansions. Under this assumption they expected allelic diversity to decrease towards the edge of the species range as a result of allelic drop-out from sequential founder effects. With this scenario in mind they concluded that the surprising abundance of unique and rare alleles that they observed in rabbits from Wellstead in WA, at the furthest edge of this range, were a result of rapid population expansion offsetting the colonisation founder effect. Our results suggest that the Western Australian rabbit population is the result of a separate historical introduction, forming a genetic cluster distinct from the Southern Victorian rabbits around the Barwon Park release site. Our strong clustering of Western Australian rabbits with rabbits from SA1 on the Eyre Peninsula of South Australia is concordant with historical accounts from Abbott (2008) that place South Australia as a major source of rabbits travelling overland to WA. We therefore postulate that, rather than being a distant migration from Barwon Park, Zenger *et al.*'s unexpectedly diverse Wellstead rabbits are in fact the product of migrants stemming from the Eyre Peninsula (where successful introductions were reported at Point Lowly (1860-64), Middlecamp (pre-1873) and Franklin Harbour (pre-1878) – see Peacock and Abbott 2013), perhaps augmented by local introductions such as that recorded at Cheyne Beach in 1886 (Peacock and Abbott 2013).

Our findings assign each of Australia's state capitals its own ancestral rabbit genotype, a pattern that bears a striking resemblance to that found by Andrew *et al.* (2017) for Australia's introduced house sparrows. Like sparrows, rabbits are typically very sedentary but capable of moderate dispersal under appropriate conditions (Parer 1982; Richardson *et al.* 2002); it is therefore unsurprising that the two species would follow a similar genetic pattern arising from early (perhaps repeated) introductions to major settlements followed by regional dispersal. Peacock and Abbott (2013) document 223 rabbit introductions to Australia during the mid-1800s, of which at least 32 were reported successful in historic articles. These span much of the country, as indicated in Figure 2a, including plausible sources for independent introductions associated with each of the ancestral clusters identified here, and the well-known release at Barwon Park in 1859 which could be a primary source of all populations within the Melbourne ancestral cluster.

The patterns of ancestry shown in Figure 2 suggest that while some populations have remained largely localised (Sites SA4, NSW3 and NSW5) others appear to have dispersed widely, resulting in the Perth, Central and Melbourne clusters. An interesting avenue of further research will be identifying the causes of this variation in dispersal, which may include local climate, landscape barriers, or factors affecting propagule pressure such as early control efforts and predator impact (as per Peacock and Abbott (2013)).

The rabbit introduction records assembled by Peacock and Abbott (2013) include both intentional and accidental releases, and undoubtedly represent only a fraction of total releases occurring at that time period. One account from c. 1820 states "probably a 100 or more distinct efforts were made by as many of the first settlers to breed rabbits in New South Wales" (*The Australasian* 20 April 1918 page 723). It is apparent that the importance of rabbits as a source of portable protein for early European settlers resulted in substantial human-mediated propagule pressure across the continent. That this pressure resulted in numerous independent population establishments is evidenced by the six ancestral clusters identified in our analyses. While the rapid colonisation of Australia by rabbits remains remarkable, it appears that natural dispersal was given a substantial head-start by association with early European colonisation.

Implications for pest management

Outside the agricultural areas, where physical control of rabbits is cost effective, management of rabbit populations in Australia currently relies on viral biocontrols to suppress numbers across a vast and sparsely populated landscape. Although both myxomatosis and RHDV were highly effective on naïve rabbit populations, mortality rates have decreased over time. Several studies (summarised by Kerr (2012)) have found evidence of adaptive resistance to myxomatosis in wild rabbits, as well as attenuation of the virus to maximise rabbit-rabbit transmission. Similarly, challenge studies by Elsworth (2012) found reduced infection rates for RHDV in some wild rabbit populations.

Understanding rabbit population structure is a key step in understanding documented variability in biocontrol resistance. In this study we have identified three primary genetic lineages of Australian rabbits, as well as the presence of highly localised lineages which may represent independent local rabbit introductions. The lineages identified here bear no apparent relationship to the distribution of RHDV resistance identified by Elsworth (2012). Elsworth (2012) found no significant resistance in the rabbits from VIC2 or QLD (Melbourne and Central clusters respectively), and high levels of RHDV resistance in rabbits from VIC1, VIC3, and SA4 (Central, Melbourne and Adelaide clusters respectively). This suggests that resistance to RHDV may have evolved several times independently at a more localised scale, however further research will be required to determine the specific genes involved and whether they differ among rabbit lineages. Where lineages exhibit strain-specific viral resistance this will aid prediction of the impact of biocontrol efforts in different regions and create opportunities for regional customisation of future biocontrol releases.

The genetic structure of invasive populations has at times been used to inform pest management strategies through the identification of 'management units' - regions between which there is minimal gene flow, due to geographic or other barriers - in which the species of interest can be suppressed or eradicated with minimal chance of reinvasion. One example of this is the identification of appropriate management units for feral pigs (*Sus scrofa*) in Australian rangelands by Cowled *et al.* (2008) who suggest that the failure of previous pig management attempts was caused by a disparity between the area of management and the geographic range of the sub-population. There has been some concern that, given the speed and scale of historical rabbit invasions, the scale of appropriate management units for this species may simply be too large for viability. Previous studies of rabbit variation in more localised areas have produced varied results. Richardson (1980) found variation in allozyme frequencies between NSW subpopulations as little as 1km apart. In contrast, Fuller *et al.* (1996) found no evidence of differentiation between nine populations of arid Queensland rabbits within a 25km radius using five allozyme loci. Phillips *et al.* (2002) found differentiation in mtDNA haplotype frequencies between rabbits from Sydney, inland NSW and Victoria which could represent our Sydney, Central and Melbourne ancestral clusters. Although our results indicate that the bulk of Australian rabbits do segregate into three geographically broad clusters, the presence of highly localised populations at Sites SA4, NSW3 and NSW5, as well as distinct cluster substructuring to the scale of sample site, suggest that the treatment of local populations as management units may indeed be viable. The conflicting results of Richardson (1980) and Fuller *et al.* (1996) indicate that the scale of appropriate management units may vary. Further studies using genome-wide sequencing techniques at regional scales will be required to identify the factors that influence the scale of localised gene flows (and thus the appropriate scale of management units).

Conclusion

Using reduced-representation sequencing techniques we have shown that Australia's introduced rabbits form three broad geographic clusters representing different ancestral lineages, along with a number of highly localised, differentiated lineages. Rather than the oft-cited single plague of rabbits dispersing rapidly from an introduction at Barwon Park in Victoria, this molecular data supports a history of multiple independent rabbit introductions across the landscape, highlighting the importance of explicitly testing popular assumptions of species invasion history. This new insight into the population structure of Australia's rabbits provides a foundation for further research to examine the impact of rabbit lineages on biocontrol resistance and optimal management units to enhance management strategies for this challenging pest species.

Chapter 4 – Reproductive strategy and gene flow in Australian rabbits

Statement of Authorship

Title of Paper	Reproductive strategy and gene flow in Australian rabbits
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
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Principal Author

Name of Principal Author (Candidate)	Amy Iannella		
Contribution to the Paper	Acquired ethics approval and funding, collected field samples. Performed DNA extraction, amplification and reduced representation library preparation. Performed sequence data filtering, SNP identification and filtering, pedigree analysis and generalised linear modelling. Interpreted outputs and wrote manuscript.		
Overall percentage (%)	90%		
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.		
Signature		Date	6 Nov 2018

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.

Name of Co-Author	David Peacock		
Contribution to the Paper	Helped in field sample collection, data interpretation, manuscript evaluation and editing		
Signature		Date	18 December 2018

Name of Co-Author	Phillip Cassey		
Contribution to the Paper	Supervised development of work, provided modelling analysis advice, helped to evaluate and edit the manuscript.		
Signature		Date	18-December-2018

Please cut and paste additional co-author panels here as required.

Name of Co-Author	Nina Schwensow		
Contribution to the Paper	Supervised development of work, helped in data interpretation, manuscript evaluation and editing		
Signature		Date	14/12. 2018

Reproductive strategy and gene flow in Australian rabbits

Abstract

Managing European rabbit (*Oryctolagus cuniculus*) abundance is internationally important, given the species' various roles as a keystone prey species and an invasive pest. To influence rabbit recruitment processes a greater understanding of the interactions between rabbit breeding strategies and disease dynamics is required. In this study we used a unique combination of capture-mark-recapture data, viral antibody assays and a pedigree derived from reduced representation DNA sequencing to provide unprecedented clarity on rabbit recruitment processes in a natural population. We found evidence of female philopatry, short-distance male-biased dispersal and high variance in reproductive output favouring heavier adults. This is congruent with earlier studies of rabbits in Europe, suggesting that differing climatic and epidemiological conditions have not affected rabbit social systems, despite demographic disruption. For the first time we have found evidence of multiple paternity in the wild and frequent mating outside of social groups. Mortality of weaned offspring was high, but survival probability was increased for offspring born in larger warrens to young dams with myxomatosis antibodies. Maternal RHDV antibodies did not affect juvenile survival, but birth month was significant, highlighting the critical role of RHDV outbreak timing in rabbit recruitment (or lack thereof) as a result of innate kitten resistance. The socially structured breeding system employed by rabbits maximises within-group heterozygosity, which likely favours the development of genetic innovations such as RHDV resistance. However, limited dispersal will delay the spread of such innovations at a landscape scale, raising the possibility that resistance has developed independently on a regional basis.

Introduction

Social structure and breeding tactics influence genetic structure

Breeding strategies and gene flow structure the genetic composition of animal populations (e.g., Wright 1946). The interplay of mate selection and dispersal regimes is particularly prominent in social species where it has been examined through empirical population studies in species as diverse as yellow-bellied marmots (Schwartz and Armitage 1980), leaf-cutting ants (Hughes and Boomsma 2004) and chestnut-crowned babblers (Rollins *et al.* 2012). In a review of empirical studies of social structure influences on genetic variation, Van Staaden (1995) concluded that "*where known social groups are compared, significant structure seems to be the rule*". This observation has been reinforced by mathematical models (e.g., Chesser 1991a; Chesser 1991b). Chesser (1991b) found that breeding strategy was of much greater importance than migration rate in determining the equilibrium value of genetic differentiation between intra-population social groups. For example, when examining the effects of female philopatry Chesser (1991a) found greater than expected

heterozygosity within social groups and that differentiation between groups was maximised when a single polygynous male bred with a group of philopatric females.

In influencing the genetic structure of a population, breeding strategies and gene flow profoundly impact the rate at which a population can adapt to new stressors. Caprio and Tabashnik (1992) simulated the evolution of insecticide resistance among finite subdivided populations and found that varying rates of gene flow had different impacts on the rate of resistance evolution, depending on the frequency of the resistance allele and the treatment heterogeneity. Hughes and Boomsma (2004) found variation in low-dose parasite resistance between different patrilines of polyandrous leaf-cutting ants, suggesting that polyandrous mating structures may result in improved disease resistance among social colonies due to increased genetic diversity. These examples demonstrate the importance of breeding strategies in influencing host-parasite co-evolution, a process which is of particular interest in the European rabbit (*Oryctolagus cuniculus*) whose populations are substantially controlled by disease (Fordham *et al.* 2012; Monterroso *et al.* 2016).

Rabbit social structure and breeding tactics

The European rabbit is a highly social mammal whose importance - as a keystone prey species on the Iberian Peninsula (Delibes-Mateos *et al.* 2007), a commercially farmed animal (Dalton *et al.* 2014), and a major ecological and agricultural pest in its invasive range (Bird *et al.* 2012; Cooke 2013; Long 2003; Mutze *et al.* 2016b) - has led to several studies of breeding strategy over the decades. The rabbit is an extremely fecund species; a single adult female can produce well over 15 kittens in a year, depending on climate (Gilbert *et al.* 1987). Rabbits reach sexual maturity at 3-4 months old, although in many regions this age is reached outside of the breeding season making the actual age of first reproduction months later. The onset and duration of the breeding season is dependent on the availability of green vegetation, temperature and photoperiod, and thus varies considerably around the world. During the breeding season females can give birth every three months, owing to a 28-31 day gestation period coupled with post-partum oestrus, although prenatal loss of litters reduces the observed number of litters produced each year (Tablado *et al.* 2009). As many as nine kittens in a litter have been observed (von Holst *et al.* 2002), although mean litter size is usually 4-6 and is correlated with rabbit mass, which tends to be higher in cooler climates (Tablado *et al.* 2009). Pregnancy rates are lower for females under 12 months old, than for females in their second or third breeding season, but the relative fecundity of older rabbits is less certain. von Holst *et al.* (1999) found increasing fecundity with age, whereas Rödel *et al.* (2009) found reduced fecundity in females after 3 years of age. The impact of density on rabbit fecundity is also uncertain. Rödel *et al.* (2004) reported reduced reproductive success at higher rabbit densities, while Tablado *et al.*'s meta-analysis (2009) could not demonstrate a density-dependent signal, possibly due to limitations in the available density data.

Rabbits live in social groups based around warrens or similar shelter. They are generally assumed to be polygynous (Surridge *et al.* 1999a), but polygynandry (Cowan 1987) and monogamous breeding pairs (Daly 1981) have also been observed, likely in response to variations in social group size and resource availability. Each sex follows a separate linear social hierarchy in which dominant rabbits have superior access to food, shelter and mates, while subordinate rabbits are impacted by aggressive behaviours, which trigger physiological stress responses leading to increased susceptibility to diseases such as coccidiosis (von Holst *et al.* 1999). As a result, dominant rabbits experience a longer effective breeding season, a longer reproductive lifespan, and reduced mortality of offspring (Rödel *et al.* 2009; von Holst *et al.* 2002; von Holst *et al.* 1999). In concert, these effects yield substantially higher lifetime reproductive success for dominant rabbits. Von Holst *et al.* (2002) observed that females of the highest ranks produced roughly four times as many adult offspring as the most subordinate females, and that females attaining the highest rank during their first reproductive season had about 60% higher lifetime reproductive success than all other females, including those whose rank subsequently rose.

Sub-adult rabbits disperse from their natal warren at around 600 - 1250 g and tend to show a pattern of male-biased dispersal and female philopatry, although the proportion of dispersers and the differential frequency between sexes both vary between sites (Künkele and von Holst 1996; Parer 1982; Richardson *et al.* 2002; Surridge *et al.* 1999b). Because adult rabbits become quite territorial during the breeding season, most breeding is generally assumed to occur within the home warren (e.g., Surridge *et al.* 1999a), although Daly (1981) did note that 7% of offspring had allozyme profiles incompatible with all known resident males. The prevalence of extra-warren breeding has not yet been examined in detail, probably because behavioural studies (e.g., von Holst *et al.* 2002) are unable to determine offspring paternity, while molecular studies using allozymes or microsatellite loci (e.g., (Daly 1981; Surridge *et al.* 1999a; Webb *et al.* 1995) have focussed on genetic structure and dispersal through measures of overall relatedness, rather than developing exact pedigrees.

The influence of warren size and quality on rabbit breeding and dispersal behaviours has been a largely neglected topic in previous studies, although Daly (1981) did note that adult rabbits rarely disperse between warrens, even if their home warren decreases in quality over time. This can lead to reduced productivity for these adults, resulting in allele frequency changes in the overall population over time as different social groups become environmentally advantaged. Rouco *et al.* (2011) provide the only explicit study of the effects of warren size, in their examination of the effectiveness of artificial warrens. They found no difference between small and large warrens in the number of juveniles produced per adult female over the breeding season.

The influence of disease

Much of our understanding of rabbit breeding ecology outlined above comes from research performed prior to the rise of rabbit haemorrhagic disease virus (RHDV) and does not specifically examine the effects of disease. Rabbit Haemorrhagic Disease (RHD) and myxomatosis have been the cause of substantial rabbit declines worldwide (e.g., Kerr 2012; Mutze *et al.* 1998; Villafuerte *et al.* 1994). RHDV1 has typically caused annual outbreaks of RHD with moderately high mortality in the later part of the breeding season, associated with new susceptible rabbits (Le Gall-Reculé *et al.* 2013; Mutze *et al.* 2014b). The influence of epizootics on rabbit survival and demographics has been examined extensively through computational models and simulations in an effort to understand and manage the frequency and intensity of associated population crashes. For example, Fordham *et al.* (2012) found that infection rate explained 80% of variance in rabbit survival (see also Barnett *et al.* 2018; Calvete 2006; Fa *et al.* 2001; Wells *et al.* 2015).

Although the effect of disease-mediated population crashes on rabbit reproductive parameters has not been tested explicitly, Richardson *et al.* (2002) investigated the effect of a population crash (due to rabbit control activities) on dispersal and found that low population density promoted an increase in juvenile dispersal distance resulting in a constant effective population size (Ne) over time. Dispersal itself can be implicated in the transmission of diseases between groups of social animals (e.g., Robertson *et al.* 2006). Although at a landscape scale flies are the principal vector for RHDV (Asgari *et al.* 1998; Schwensow *et al.* 2014), and mosquitos for myxomatosis (Day *et al.* 1956), interactions between rabbits are likely to contribute to viral spread between individuals locally. Jennings and Mutze (2017) examined sub-adult dispersal activities as a potential source of inter-warren disease transmission, but extra-warren breeding is another source of warren-to-warren transmission that has not previously been examined in detail.

The role of very young rabbits in disease development and transmission has previously been studied, showing that they are highly resistant to RHD infection until about 8 weeks old, typically experiencing only subclinical symptoms (Ferreira *et al.* 2004; Xu and Chen 1989), although some newer strains and serotypes of RHDV appear to have increased infectivity towards young rabbits (Dalton *et al.* 2012; Mutze *et al.* 2014b; Neave *et al.* 2018). Rabbits that recover from RHD produce antibodies against the virus which confer lifelong protection from further infection by related serotypes, given repeated exposure to the disease which boosts antibody titre (Ferreira *et al.* 2008). For dams seropositive to RHD these antibodies pass across the placenta to embryos, reducing severity of RHD infections until about 12 weeks of age (Robinson *et al.* 2002). Similarly, maternal myxomatosis antibodies can be detected in kittens until six or seven weeks old (Fenner and Marshall 1954; Kerr 1997), although whether these antibodies confer protection from the virus is uncertain (Fenner and Marshall 1954; Sobey and Conolly 1975). The impact of dam antibody titres on offspring survival under natural field conditions is yet to be examined for either virus.

Understanding the epidemiological and demographic processes involved in rabbit recruitment and gene flow is critical to inform the development of models that assess the impact of rabbit management strategies, as well as the impacts of disease. While this important species has been well studied over the decades, questions remain to be answered. This study used a unique long-term dataset from a natural population, combining capture-mark-recapture data, viral antibody assays and a pedigree derived from reduced representation DNA sequencing to provide unprecedented clarity on rabbit recruitment processes. Specifically, it addressed the following questions:

- Are rabbit breeding and dispersal parameters in a South Australian temperate population characterised by annual disease-mediated population crashes comparable to those observed elsewhere?
- How frequent is extra-warren breeding?
- Does warren size affect reproductive output, juvenile survival or dispersal regimes?
- Does the presence of antibodies to myxomatosis or RHDV in a female increase the chance of survival in her offspring?
- How might socially mediated patterns of gene flow within rabbit populations impact the spread of genetic innovations such as RHDV resistance?

Methods

Study population

Turretfield Research Station, 50 km north of Adelaide, South Australia ($34^0$33'S, $138^0$50'E) is the site of an on-going 22-year rabbit monitoring study. It has a Mediterranean climate and contains around 19 warrens (mapped in Figure 1) across a size gradient of approximately 1-30 holes (variable over time). The closest known neighbouring warrens are 1 km away. Rabbits from this population have previously been shown to possess considerable resistance to RHDV infection (Elsworth *et al.* 2012). Rabbits were live-trapped using wire treadle cage traps baited with carrot for 4-5 consecutive days at 8-12 week intervals. For each individual caught on a trip, the capture warren, sex, weight, reproductive status, flea abundance index (scale of 0-3) and presence of clinical myxomatosis were noted. Date of birth was estimated for individuals caught for the first time under 1250g, based on an estimated growth rate of 10g/day (Peacock and Sinclair 2009). 1-2ml blood samples were taken on each occasion and antibodies to RHDV and myxomatosis were tested using ELISA, following Capucci *et al.* (1991, 1997) and Cooke *et al.* (2000). Each rabbit was tagged with a numbered plastic ear-tag on their first occasion of capture. Based on the proportion of tagged carcasses at this site, over 95% of adults in the population are caught at least once, but capture rate is low for young kittens due to their high mortality (Mutze *et al.* 2014b). For this study, ear tissue samples were taken from captured rabbits during the regular trapping throughout 2013 and 2014 and stored in 70% ethanol, under University of Adelaide Animal Ethics Approval Number S-2014-059. All previous capture records and serological data for these rabbits were made available for the project by Biosecurity SA.

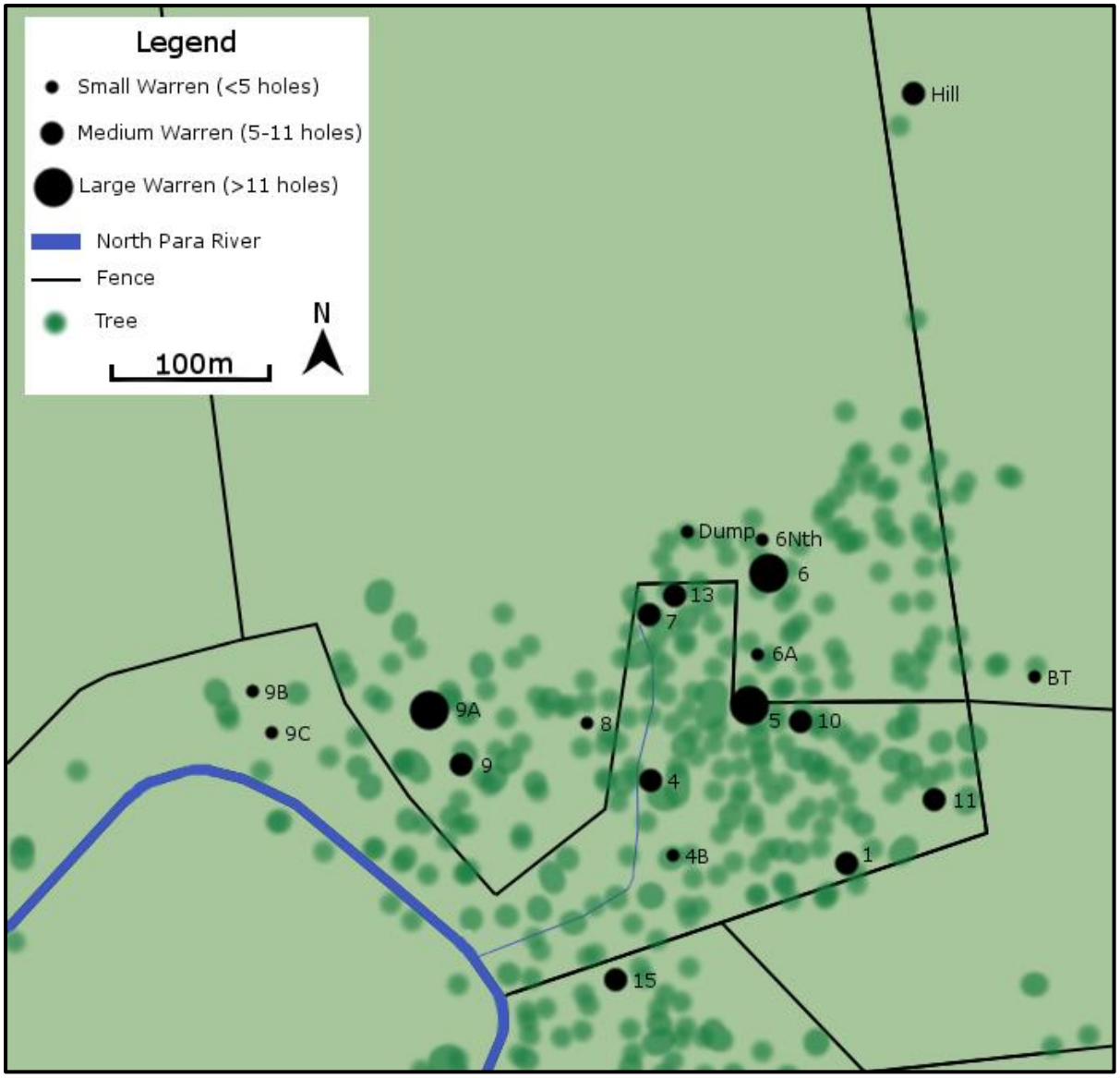

Figure 1: Map of rabbit warrens at Turretfield Research Station, South Australia. Warrens are shown as black dots with size indicating the number of warren entrances, and labelled with warren name.

DNA extraction and sequencing

DNA was extracted from approximately 6mm^2 of each rabbit ear tissue sample using the Gentra Puregene tissue extraction protocol and eluted in 75µl TLE buffer. Extract was quantified using a Picogreen dsDNA quantitation kit on either a Biotek Synergy HT or a BMG Labtech PHERAstar plate reader and 200ng was used to prepare a double digest GBS library following the protocol of Poland *et al.* (2012), using New England BioLabs PstI-HF as enzyme 1 and New England BioLabs MspI-HF as enzyme 2. PCR product was purified using the Invitrogen PureLink Quick PCR Purification Kit. 96 samples including a negative control were pooled per run of 75 cycle single-end sequencing on an Illumina NextSeq 500 at the Australian Genome Research Facility.

SNP determination

Raw sequence reads were filtered for quality (sliding window phred score limit of 10) and adapter presence, trimmed to 40bp and demultiplexed using the process_radtags program from the software Stacks v1.34 (Catchen *et al.* 2013). Files were checked for quality using FastQC. Reads were then mapped to the rabbit genome OryCun2.0 (available from the National Center for Biotechnology Information (NCBI) at www.ncbi.nlm.nih.gov/genome/316?genome_assembly_id=203429) using the bwa aln/samse functions from the software Burrows-Wheeler Aligner (Li and Durbin 2009). The Stacks v1.44 pipeline ref_map was then used to call SNPs with a minimum stack depth of 10, minimum minor allele frequency of 0.2 (to maximise pedigree informativeness) and minimum of 85% of samples represented (to reduce the impact of missing data created by null alleles). Files were checked for quality at each stage using FastQC. Based on the Stacks sumstats output, sex-linked loci (which mapped to the X-chromosome (NC_013690.1) or had no male heterozygotes) were removed from the dataset. Rabbits with >75% missing loci were removed from the dataset. Locus subsets were used to reduce the impact of remaining missing data during pedigree construction as outlined below.

Identity analysis

Identity analysis was performed using Cervus 3.0.7 (Kalinowski *et al.* 2007) to identify any unintentional duplicates in the data because very occasionally rabbits lose their identifying ear tags and are treated as new when subsequently captured. Fuzzy matching allowed up to 50 mismatched loci (control duplicates had 6-34 mismatches recorded) and duplicates were considered plausible where sex and approximate age were compatible. One member of each plausible duplicate was removed from the dataset.

Pedigree construction

For parentage analysis using likelihood methods with Cervus 3.0.7, the final SNP dataset was used to create seven smaller replicate datasets. Five datasets each comprised 700 loci selected randomly using a custom script. One dataset comprised the 350 loci with the least missing data. The final dataset comprised the 310 loci that were sequenced for rabbit Y2720 - a 3-year-old rabbit that was poorly sequenced but suspected to be a dominant breeder and therefore important to include in the analysis. Candidate parents for each sampled rabbit were identified based on capture records. Candidate parents had to be of the correct sex, born at least 6 months before the putative offspring and have been recorded weighing over 1500g.

To calculate critical values for likelihood ratios parentage simulations were performed in using Cervus for 10,000 offspring; assuming 80% of candidate parents sampled (a conservative estimate based on Mutze *et al.* (2014b), locus mistype rate of 0.01 (based on duplicate genotyping mismatches) and a minimum of 150 typed loci required for sample inclusion. Parentage analysis was then performed for each of the seven replicate datasets

using Cervus, with confidence assigned based on these simulations. Offspring-dam-sire trios and offspring-parent pairs that were allocated with confidence were compared among the replicate datasets. Trios were considered supported by consensus if supported by at least three of the seven replicates, or at least five replicates where an alternative trio was also supported. Pairs were considered supported by consensus if supported by at least four of the seven replicates, or at least six replicates, where an alternative pair was also supported. Trios and pairs involving the rabbit Y2720 were accepted if supported by that data subset with no alternative proposed by other datasets.

Analysis of breeding system

PedigreeViewer (Kinghorn and Kinghorn 2015) was used to display consensus relationships as a pedigree chart and to calculate pedigree-based inbreeding values. Wright's F_{IS} was calculated for each warren using the software package GenAlEx 6.503 (Peakall and Smouse 2012) to measure the heterozygosity reduction compared to Hardy-Weinberg expectations of random mating, as an indication of breeding structure. The number of mates that produced offspring with each adult was calculated to determine the incidence of polygamy. Offspring born to the same dam with an estimated birth date within 15 days of each other were considered likely to be from the same litter. Sires were compared among littermates to determine the incidence of multiple paternity.

Offspring were categorised as 'surviving to reproductive age' or 'dying young' based on inference from capture data. Rabbits were considered survivors if caught weighing >1500g, or dead if a tag or carcass was found before the rabbit could have reached 1500g (based on a growth rate of 10g/day; Peacock and Sinclair 2009). Rabbits that were not recovered alive or dead after 1500g were assumed to have died before having an opportunity to reproduce. Migration offsite or failure to trap adult rabbits surviving onsite are alternative reasons that a rabbit may go undetected; Wells *et al.* (2018) estimated that capture probabilities for this site in 2013 and 2014 varied seasonally between 10% and 60%. However, the very high rate of genuine mortality in rabbits makes error from these sources likely to be relatively small (Cowan 1987), especially when rabbits remain uncaptured over several occasions.

Warrens were categorised as small (less than five holes), medium (five to 11 holes) or large (12 or more holes). These categories reflect field observations that warrens with less than 5 holes can be ephemeral, while the few warrens with over 11 holes take up substantially more land area and become dominant landscape features. The effect of age, sex, maximum recorded weight and warren size on the number of offspring detected for each adult rabbit were tested using negative binomial generalised linear modelling (GLM) in the statistical environment R (version 3.5.1, R Core Team 2016). The effect of dam age, presence of dam antibodies to myxomatosis and RHDV, natal warren size and birth month on offspring survival probability were tested using binomial GLM within the R computing package MASS (version 7.3-50, Venables and Ripley 2002). Births in November through to February were

combined into the category ‘summer’, and March through to June in the category ‘autumn’ because breeding and survival in these months was low and resulting monthly survival sample size was < 5. All combinations of factors, not including interactions, were tested. Models with ΔAIC < 4 were averaged using the ‘full average’ coefficients from the R package MuMIn (version 1.42.1, Barto 2018). Relationships between offspring survival and significant variables were visualised using the R package visreg (version 2.5-0, Breheny and Burchett 2018).

Inter-warren dispersals on the site were quantified for the rabbits in this study using capture-mark-recapture records. The warren at which a rabbit was most commonly recorded after maturity was considered the ‘home warren’ and counted as a dispersal if it differed from the first recorded warren as a juvenile (the ‘natal warren’). Pedigree data was used to assess the breeding success of dispersed rabbits and the frequency of inter-warren breeding. Chi-squared tests were used to assess the impact of sex on dispersal, and of warren size on dispersal and inter-warren-breeding, using expected values weighted by warren population size.

Mean pairwise relatedness of rabbits at each warren was calculated using the program Coancestry v 1.0.1.9 (Wang 2011). To determine which of the seven relatedness estimators implemented in Coancestry is most suitable for this dataset, all estimators were first calculated for a test dataset comprising rabbits from just two medium-sized warrens in this study (Warrens 7 and 15) using allele frequencies calculated from the full dataset. The test dataset included 325 rabbit dyads which, based on pedigree reconstruction, included 39 offspring-parent dyads (pedigree relatedness (r = 0.5), 61 full sibling dyads (r = 0.5), 30 half-sibling dyads (r = 0.25) and 195 dyads with no known relationship (r = 0). The relatedness estimation values (R) were plotted against the expected pedigree-based values (r) to choose the estimator with the closest fit across all relationship types, which was then calculated for the entire dataset. Mean R was tested for difference between sexes at each warren with at least 4 members of each sex, using 1000 bootstraps to test the interaction between sex and family in determining social groups. Rabbits with less than ten R values above 0.125 across all dyads were identified as potential immigrants from outside of the Turretfield population if not otherwise indicated by the capture-mark-recapture record (i.e., not known as a kitten on site).

Results

Pedigree construction

The final dataset included 461 rabbits and 4699 SNP loci. Identity analysis uncovered three plausible matches in which a rabbit may have lost a tag and later been re-tagged as ‘new’ (0.65%), and an additional two ‘matches’ which may represent littermates (e.g., a male and a female of the same age and warren).

Likelihood-based parentage analysis with Cervus yielded 277 strongly supported offspring-sire-dam trios and a further 97 parent-offspring pairs. Y2720 was assigned three offspring, all with the same dam and with three or less mismatching loci, suggesting that the reduced stringency for this sire did not introduce poorly supported relationships. Polyandry and polygyny were both common in this study, with 70% of dams and 62% of sires detected with offspring produced by more than one mate. 13% of dams and 15% of sires had offspring with more than three mates, up to a maximum of seven for the females (Figure 2). Among all offspring in this study we found 650 full sibling dyads, including 143 with estimated birthdates within 15 days of each other, considered likely to be from the same litter. A further 17 dyads were identified as maternal half-siblings with birthdates within 15 days, indicating the presence of multiple paternity.

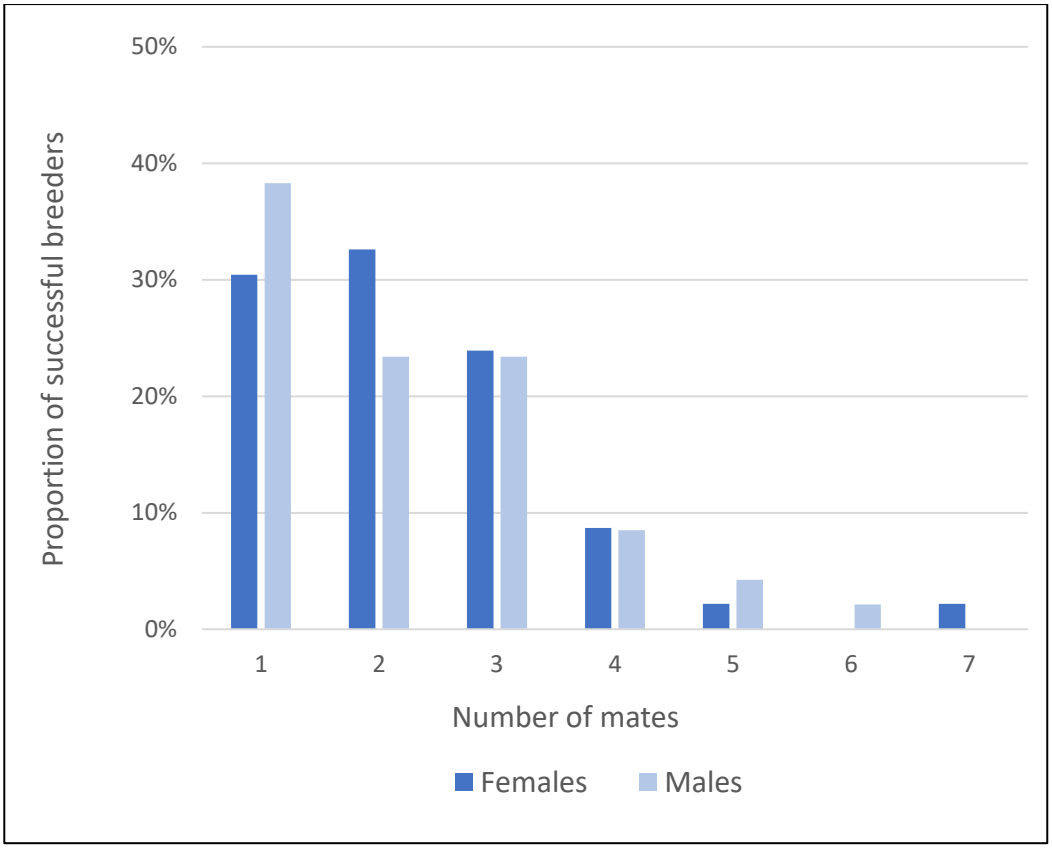

Figure 2: Prevalence of polygyny and polyandry in the wild rabbit population at Turretfield, South Australia in 2013 and 2014.

Reproductive output

Reproductive success was distributed unequally among mature rabbits. Of 90 mature males and 61 mature females, only 49% (44) and 67% (41) respectively were found to have offspring in this study. Maximum rabbit weight was the only significant factor in the GLM assessing total reproductive output (Table 1a), with rabbits below 1750g producing less

offspring on average (Figure 3a). Rabbit age, sex and home warren size did not have any significant impact on reproductive output.

19% of the 372 offspring from 2013 and 2014 sampled in this study were categorised as surviving to reproductive age. Parentage data indicated that of the reproducing rabbits only 56.8% (25) of males and 58.5% (24) of females had any offspring in 2013 or 2014 that survived to reproductive age. The averaged model for natal factors impacting offspring survival, summarised in Table 1b, included significant effects for dam age, presence of myxomatosis antibodies in dam, natal warren size and month of birth, but not the presence of RHDV antibodies in the dam. Significant predictors of offspring survival are presented as summarised data in Figure 3b-c, with modelled survival probabilities visualised in Figure 4 for comparison. In this model, survival probability decreased significantly with dam age (Figure 4b) but increased dramatically when the mother carried antibodies for myxomatosis ($P < 0.0001$, Figure 4c). Survival probability was lower for rabbits born in medium ($P = 0.002$) or small warrens ($P = 0.005$), than for those born in large warrens (Figure 4d). Survival probability was higher for rabbits born in August or September, while no rabbits born during April or between November and February survived to maturity in this study (Figure 3b, Figure 4a).

Inbreeding and Dispersal

11 of 378 offspring (2.9%) were identified with non-zero inbreeding coefficients based on pedigree data. Five of these had values of 0.25, apparently being the product of two dams that backcrossed with their own fathers. This finding is likely to underestimate the occurrence of inbreeding within the population because the pedigree is incomplete, spanning only two years, and therefore will not account for shared ancestry deeper than two generations or involving unresolved familial links. The mean F_{IS} for breeding groups (warrens) in this population was -0.130 (standard deviation 0.042).

30 female (12.7%) and 73 male (33.0%) rabbits in this study were detected as inter-warren dispersers, being most commonly trapped as adults at a warren other than their warren of birth. This represents a significant effect of sex ($\chi^2=26.991$, d.f.=1, $P < 0.00001$), with rabbits displaying male-biased dispersal and female natal philopatry. 14 rabbits, including just one female, were recorded as dispersing more than once during their lifespan. Noting the spatial constraints of the Turretfield warren complex (575m x 325m), dispersals were typically less than 225 m to a warren (Figure 5). We did not detect any association between dispersal and the size of either a rabbit's natal warren ($\chi^2=1.889$, d.f.=2, $P = 0.3889$) or their adult home warren ($\chi^2=1.352$, d.f.=2, $P = 0.5087$).

Table 1: Model-averaged coefficient outputs for a) rabbit reproductive output negative binomial GLM, with reference sex as female and reference warren size as large and b) offspring survival binomial GLM. Note that no kittens born during summer survived in this study, resulting in overinflated standard error for this month. Reference birth month was October, reference antibody status for myxomatosis and RHDV were negative and reference warren size was large. Pr(>|z|) represents the two-tailed P-value, significant terms at $\alpha = 0.05$ are marked with a *. Max Weight is the rabbit's maximum recorded weight in grams, Age is the rabbit's age in months at Dec 1st, Dam Age is the dam's age in months at offspring's date of birth.

a) Reproductive Output Model-averaged coefficients:

	Estimate	**Std. Error**	**Adjusted SE**	**z value**	**Pr(>|z|)**
(Intercept)	-27.530	8.179	8.221	3.349	0.001*
Max Weight	0.026	0.008	0.008	3.054	0.002*
Max Weight^2	0.000	0.000	0.000	2.657	0.008*
Age	0.007	0.022	0.022	0.334	0.738
Age^2	0.000	0.000	0.000	0.623	0.534
Warren-Medium	0.001	0.110	0.111	0.007	0.994
Warren-Small	0.118	0.268	0.268	0.439	0.660
Sex-Male	0.026	0.124	0.124	0.212	0.832

b) Offspring Survival Model-averaged coefficients:

	Estimate	**Std. Error**	**Adjusted SE**	**z value**	**Pr(>|z|)**
(Intercept)	-3.126	1.395	1.401	2.231	0.026*
Birth Month Jul	0.798	0.942	0.947	0.842	0.400
Birth Month Aug	2.760	0.917	0.921	2.996	0.003*
Birth Month Sep	2.749	1.015	1.021	2.694	0.007*
Birth Month Summer	-12.778	1356.822	1363.919	0.009	0.993
Birth Month Autumn	-0.597	0.894	0.899	0.664	0.507
Dam Age	-0.100	0.024	0.024	4.125	0.000*
Dam Myxo Positive	4.531	0.924	0.928	4.880	0.000*
Warren-Medium	-1.488	0.524	0.526	2.827	0.005*
Warren-Small	-2.435	0.863	0.868	2.806	0.005*
Dam RHDV Positive	0.542	0.973	0.976	0.555	0.579

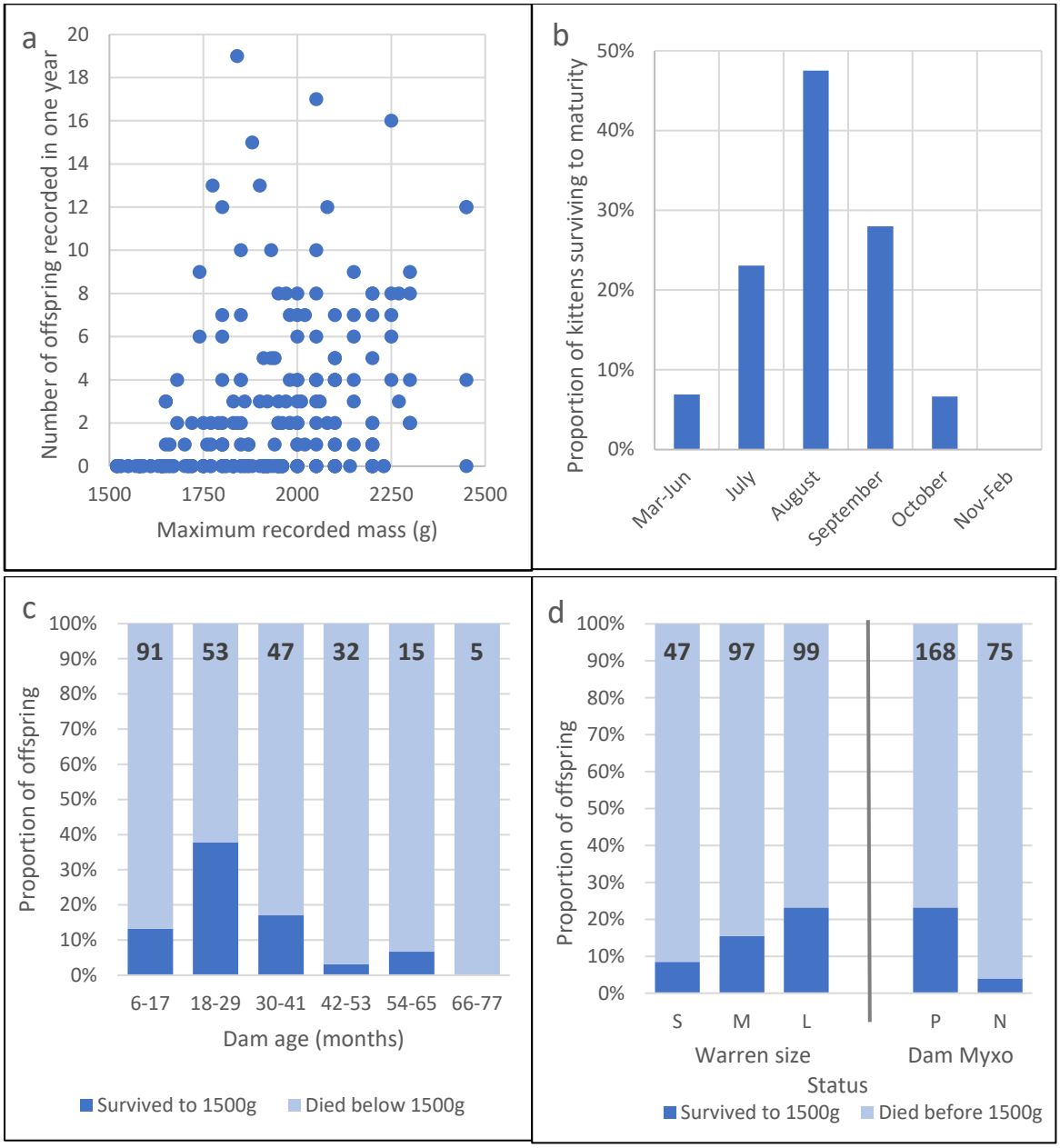

Figure 3: Significant predictors of reproductive output and offspring survival. a) Total number of offspring produced is lower for rabbits with maximum recorded weight under 1750g. b) Kittens at Turretfield, South Australia are more likely to survive if born from July to September. c) Offspring survival peaks in the dam's second breeding season and thereafter declines with age. Dam age brackets are clustered into 12-month groups approximately according to breeding seasons, n for each bracket is inset on bars. d) Larger warrens and dam myxomatosis antibodies increase survival in rabbit offspring. Warren size categories are: S = small (1 - 4 holes), M = medium (5 - 11 holes), L = large (12+ holes). Dam myxomatosis antibody statuses are P = positive, N = negative.

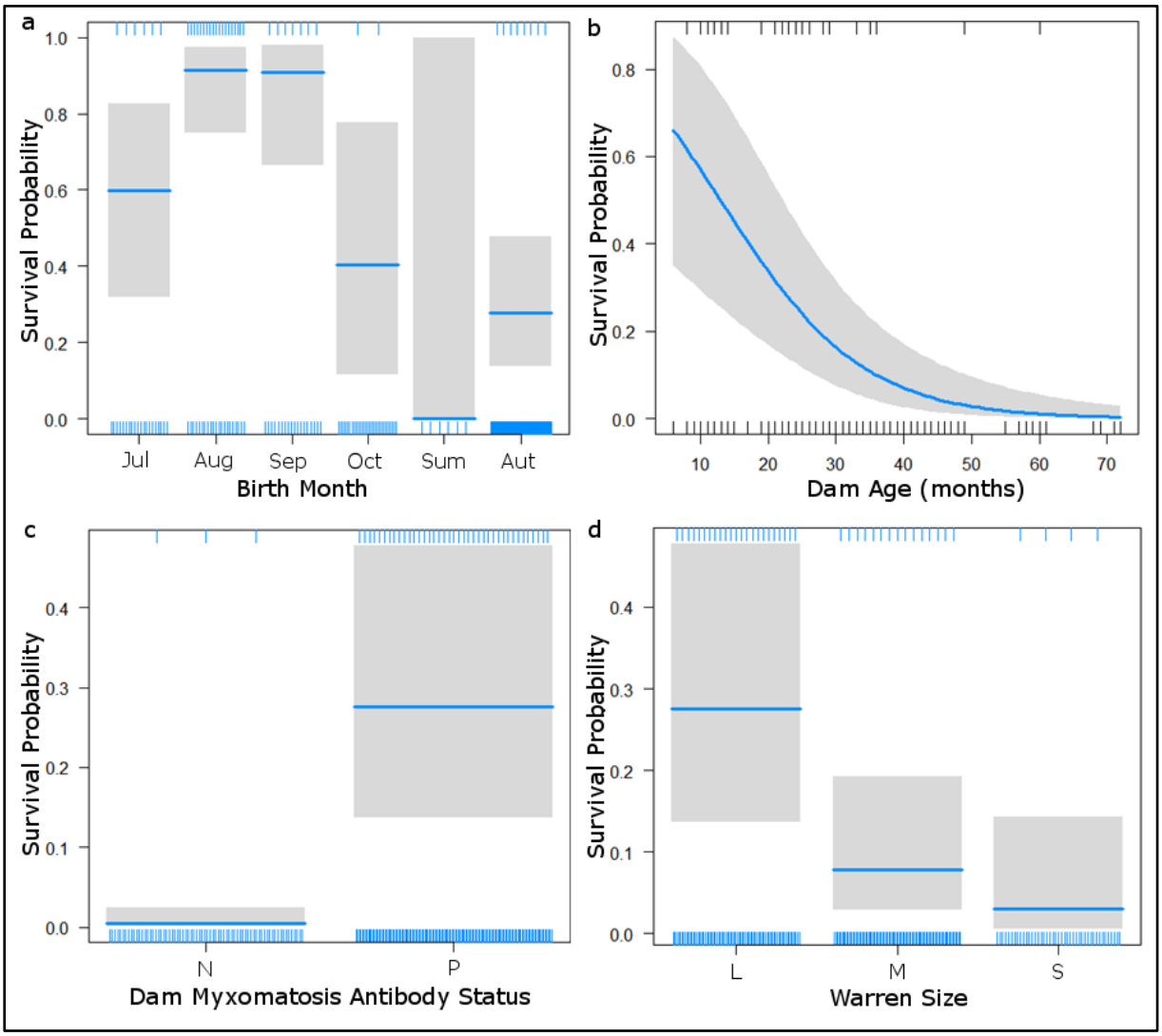

Figure 4: Visualisation of significant predictors of rabbit offspring survival in the most supported generalised linear model. 95% confidence intervals are plotted in grey, blue dashes along the top and bottom of graphs indicate data points for survival and death before maturity respectively. a) The influence of birth month on offspring survival probability. Summer (Sum) represents November - February, Autumn (Aut) represents March - June. b) The influence of dam age on offspring survival probability. c) The influence of dam myxomatosis antibody status on offspring survival probability (N = negative, P = positive blood antibody status) d) The influence of natal warren size on offspring survival probability. Warren size categories are: S = small (1 - 4 holes), M = medium (5 - 11 holes), L = large (12+ holes).

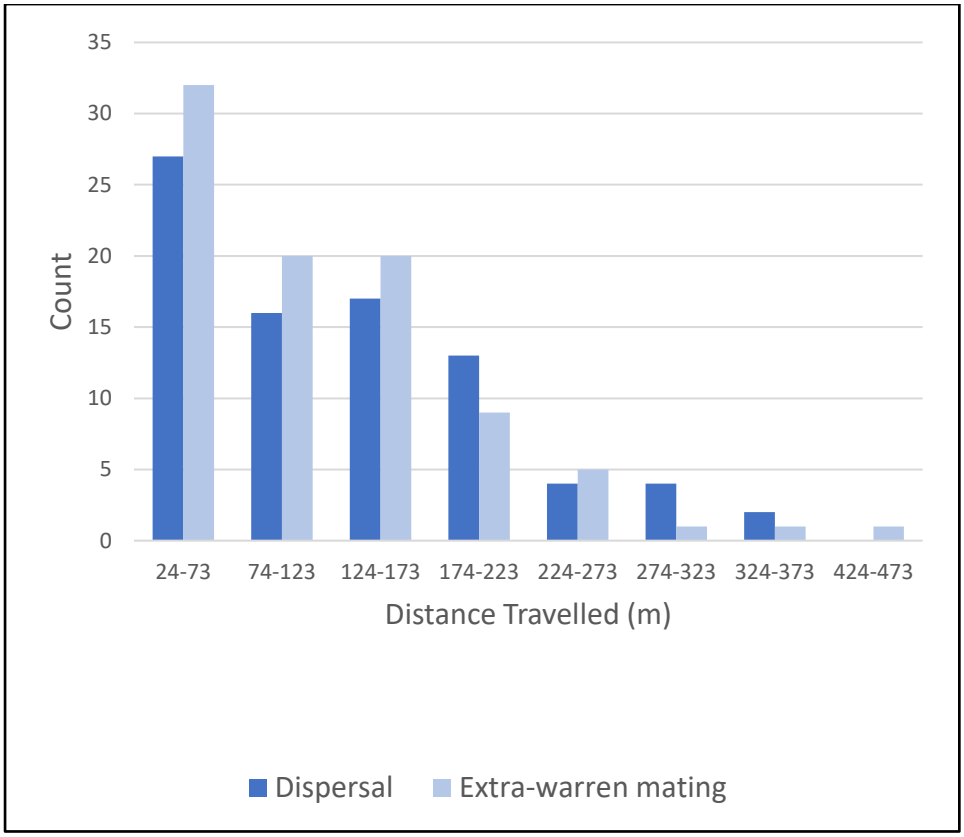

Figure 5: Frequency distribution of rabbit inter-warren movements at Turretfield, South Australia. Dispersal distance (dark blue) is from natal warren to home (most common) warren at maturity (not including non-dispersers). Extra-warren mating distance (light blue) is calculated from a sire's home warren to the offspring's natal warren.

A maximum of two breeding males and two breeding females were detected at small warrens during this study, while up to four females and eight males bred at medium-sized warrens. The largest warren (Warren 6) was home to ten breeding females serviced by 12 males. Extra-warren breeding was common among adult males, with 32.2% of offspring assigned to males from outside their natal warren. Like dispersal, inter-warren breeding was most common between closely neighbouring warrens (Figure 5). Neither the size of a sire's home warren (χ^2=2.649, d.f.=2, P = 0.2660) nor his offspring's natal warren (χ^2=5.011, d.f.=2, P = 0.0816) were significant predictors of extra-warren breeding. Males who bred outside their home warren did not have a significantly different number of total offspring than males who bred only within their home warren (χ^2=8.481, d.f.=4, P = 0.075). Among those males who bred outside their home warren, there was an inverse relationship between total number of offspring and the proportion of those offspring from other warrens (R^2 = 0.32).

The most appropriate relatedness estimator for this dataset was the moment estimator proposed by Wang (2002) which approximated expected values in our pedigree-based test data across all relationship categories. The dyad and trio maximum likelihood methods performed better for unrelated rabbit pairs but underestimated the relatedness of sibling

and parent-offspring pairs. Mean within-warren relatedness (R) using the Wang (2002) method ranged from 0.016 (Warren 8) to 0.336 (Warren 7), indicating that warrens are inhabited by family groups but are not exclusive to nuclear families. Average relatedness of around 0.25 (equivalent of a second degree relative such as a half-sibling or niece/nephew) could be achieved by multiple cohabiting families or a single family with several unrelated additions. Mean relatedness was generally higher for females within a warren than for males, as shown in Figure 6. This trend was reversed in two of the smallest warrens, Dump and Bottle Tree (BT).

Four rabbits out of 439 (0.9%) were identified as having less than ten relatives (with calculated $R > 0.125$) in this dataset. Three of these rabbits were first caught weighing between 1500g and 1700g, weights typically indicative of mature rabbits at this site. These three rabbits all had no parents or siblings identified in the pedigree (although two had offspring), supporting their status as potential immigrants. The fourth potential immigrant was first caught as a juvenile at 500g and has no identified relations in the pedigree. Rabbits have been recorded as just beginning to disperse around this size (Richardson 2002), making the immigrant status of this individual uncertain but not implausible.

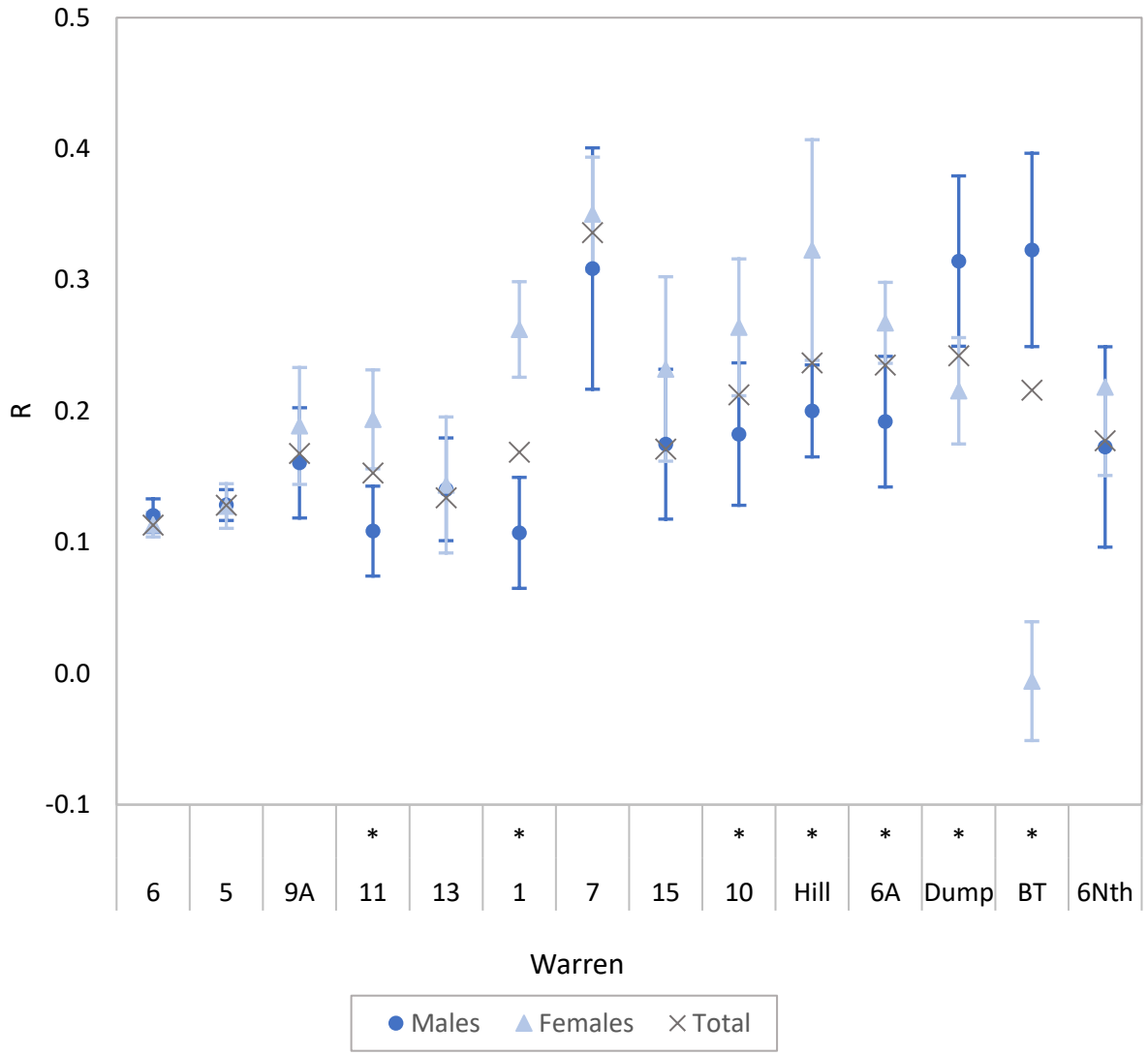

Figure 6: Mean relatedness (R) among rabbits by warren. Warrens are displayed left-to-right in order from largest (>30 holes) to smallest (1-2 holes). Crosses indicate mean R across all rabbits in the warren. Dark blue dots and error bars indicate mean and 95% confidence interval for males, light blue triangles and error bars represent females. Warrens with a significant difference in R between sexes as assessed through bootstrapping are marked with a *.

Discussion

Rabbit breeding strategies

In this study we report the first evidence of multiple paternity in wild European rabbits through the presence of maternal half-siblings with approximately matching birthdates. Multiple paternity has previously been observed in wild pygmy rabbits (Falcón *et al.* 2011), and snowshoe hares (Burton 2002), but has only been produced in laboratory experiments

in the European rabbit (e.g., Dziuk 1965). As induced ovulators (Heape 1905), the window of time for multiple mating in rabbits is likely to be small, but Dziuk (1965) demonstrated that multiple mating during the 10 hours between copulation and ovulation can produce litters of mixed paternity. The proportion of offspring attributed to the second male was lower than the first and decreased when there was more than 1 hour between copulations. In a field situation we might expect dominant males to mate with receptive females first, and subordinates to approach later, thus siring fewer offspring if any at all.

Breeding was generally polygynandrous in our South Australian pedigree, which is consistent with breeding strategies observed elsewhere using observational studies, allozymes and microsatellites. Despite the frequency of multiple mating in this system, reproductive output was not indiscriminate with half of the mature males and a third of the mature females having no detected offspring. The reduced reproductive output for rabbits under 1750g found in this study is consistent with literature recording the social dominance of large rabbits (Daly 1981; von Holst *et al.* 1999). Our lack of model support for rabbit age impacting on the number of offspring produced suggests that size may be more important than age for achieving social dominance, although observational studies would be required to confirm this. For females the influence of mass may also reflect a greater energetic capacity to support offspring. Tablado *et al.* (2009) and Lello *et al.* (2005) observed that heavier females were able to produce larger litters, while frequent resorption of litters has been observed and may be associated with resource limitation (Brambell 1948; Myers *et al.* 1994; von Holst *et al.* 1999). Another likely explanation for the importance of body mass is that older rabbits are almost universally seropositive for RHDV at this site, while newly matured rabbits at 1500 - 1600g have an increased chance of dying from RHDV during the breeding season. In this scenario, lower reproductive output resulting from senescence at older age categories may counteract the seropositivity effect, explaining the lack of model support for age overall.

Dispersal and Inbreeding

Rabbit dispersals from natal warren to adult home warren in our recapture data were predominantly to adjacent warrens and dispersal distance rarely exceeded 225m (Figure 5). This result mirrors the findings from other rabbit populations which indicate that dispersals are generally to neighbouring pre-established warrens, with distances of >500m very uncommon (Jennings and Mutze 2017; Parer 1982; Richardson *et al.* 2002). We can therefore conclude that rabbits are typically only short-range dispersers, although larger distances are possible, as indicated by four potential immigrants found in our study population. Our low total proportions of dispersing rabbits (12.7% of females and 33% of males) as compared to previous studies are likely to be caused by the high mortality of juveniles prior to dispersal in the RHDV outbreaks that occurred during both years of our study.

We had hypothesised that large warrens may act as source populations exporting subdominant rabbits to smaller, lower-quality neighbouring warrens where social pressure may be less intense due to the smaller hierarchy (von Holst 1998). Surprisingly, no relationship was observed between dispersal and the size of either source or target warren. It may be that dispersal is an innate instinct for inbreeding avoidance rather than driven by social pressure, as concluded by Künkele and von Holst (1996), and is guided by proximity rather than the quality of neighbouring warrens.

Our recapture data indicated a pattern of male-biased dispersal and female philopatry, which was largely reflected in estimates of within-warren relatedness (R) by gender (Figure 6) and is in agreement with existing literature (Richardson *et al.* 2002; Surridge *et al.* 1999b). We consider the high relatedness of males and low relatedness of females found in warrens Bottle Tree (BT) and Dump to be artefacts of the low sample size in these warrens where litters of predominantly male offspring appear to have skewed relatedness in our sampling year. Mean relatedness is noticeably lower in the two largest warrens (Warrens 5 and 6). As described by Parreira and Chikhi (2015) this may indicate that more than one effective social group is present in these sprawling warrens, such that a Wahlund effect is created by analysing them as single groups. Indeed, these two warrens were among the highest F_{IS} values (-0.0835 and -0.0719 respectively) which supports this hypothesis. Subdivision of social groups inhabiting very large warrens would also fit with the observations of Rouco *et al.* (2011) and Surridge *et al.* (1999a) for optimal group size in rabbits.

Heterozygosity excess within social groups - revealed as negative F_{IS} values - is a common theme among studies of genetic structure in social species, even in the absence of inbreeding avoidance mechanisms (e.g., Chesser 1991a; Pope 1992; Schwartz and Armitage 1980; Storz 1999). Parreira and Chikhi (2015) and Chesser (1991b) show that this heterozygosity excess is an intrinsic property of complex social group structures, although its extent differs among breeding systems. As predicted by Chesser (1991a, b) and Parreira and Chikhi (2015) we found negative F_{IS} in this population, indicative of a socially structured breeding strategy. The mean F_{IS} value of -0.130 is highly negative, fitting within the lower range of monogamous or polygynandrous social structures with female philopatry based on Parreira and Chikhi's (2015) simulations.

Although F_{IS} is not considered a reliable indicator of the presence or absence of inbreeding in a socially structured population, our estimation of an explicit pedigree did enable the detection of inbreeding within our sample. While inbreeding was infrequent, affecting just 3% of offspring, its presence suggests that rabbits do not avoid inbreeding directly. Instead, the low rate of inbreeding appears to be a product of the socially structured breeding system and male-biased dispersal. While rabbit lifespans are generally short, 39 males at this site (since records started in 1996) lived from 4 – 5.8 years, which could leave them breeding with a social group full of daughters as a result of male-biased dispersal. However,

of the 18 oldest males in this study, all at least 3 years old, we found that, based on our pedigree, none had bred with known relatives. Five of these rabbits had no detected offspring within their final two years, 13 bred outside their home warren and seven dispersed a second time between two and four years of age. This second dispersal may be a mechanism of inbreeding avoidance or a result of unfavourable social pressure.

The extent of extra-warren breeding detected in this population (one third of offspring sired by extra-warren males) is surprising, given that rabbits are generally presumed to breed only within their social group (Surridge *et al.* 1999b), although Daly (1981) did find that 7% of offspring were incompatible with all known males in their group. This result suggests that social contact of adults between warrens for mating purposes is an underappreciated avenue of disease transmission and gene flow in rabbits. The lack of association between extra-warren breeding and either warren size or total reproductive output suggests that this breeding is opportunistic rather than strategic. However, opportunistic extra-warren breeding is relatively more important for males with low reproductive output, presumably subordinates, for whom these may be their only chance of successful reproduction.

Offspring survival

Extremely high mortality in rabbit kittens is well recorded, with the proportion of offspring reaching maturity averaging 5.5%, but reaching 14.9% in some years (von Holst *et al.* 2002). Given that von Holst *et al.* (2002) recorded mortality of unweaned kittens in the nest at around 40%, these figures correspond to an average of 9% and up to 24.8% survival of weaned juveniles. Overall survival rates of weaned juveniles in our study were comparable, at 19%, but were not evenly distributed across parents. Only 28% of adult males and 39% of adult females had any offspring survive to reproductive age, a result substantially smaller than found by von Holst *et al.* (2002) and indicating a high variance in lifetime reproductive success. Our investigations into this variation, through maternal influences on offspring survival, yielded a mixture of expected and unanticipated results, which we will discuss here in detail.

Although, like Rouco *et al.* (2011), we found that warren size did not affect the total reproductive output of adult rabbits, we did find an increase in offspring survival in larger warrens (Figure 3d, Figure 4d). This result follows expectations given that large warrens are likely to provide more extensive shelter from predators and unfavourable weather, benefiting kitten survival.

More surprisingly, our models found no support for an effect of dam RHDV antibodies on kitten survival, despite strong experimental support for the protective capacity of RHDV maternal antibodies (Robinson *et al.* 2002). Robinson *et al.* (2002) found that survival of experimentally inoculated kittens was increased with larger dam RHDV antibody titres, but found no effect of kitten titre, concluding that maternal antibodies in kittens can be protective even at levels too low for ELISA detection. The significance of birth month in our

offspring survival model hints at an explanation for the low impact of maternal RHDV antibodies in this field situation. Kittens born in August and September (months associated with increased survival in this study; see Table 1b, Figure 3b and Figure 4a) would have experienced their first RHDV1 outbreak between 2 and 8 weeks old, when juvenile resistance is strongest (Ferreira *et al.* 2004). These resistant kittens have a high likelihood of surviving exposure to the disease, but typically still seroconvert (Ferreira *et al.* 2008), granting lifelong immunity to RHDV1. This process explains the significantly higher survival probability for kittens born just prior to an outbreak, regardless of maternal antibodies. Previous studies have reported a greater chance of survival for offspring born early in the breeding season, suggesting less competition from other offspring and more time to grow before a cold winter as explanatory reasons (Daly 1981; Gilbert *et al.* 1987; Rödel *et al.* 2009). It appears the influence of viral dynamics in this population (and perhaps the mild winters in South Australia) outweighs those factors, favouring offspring born in the middle of the breeding season. Importantly, since this study was conducted RHDV2 has largely replaced the descendants of the Czech-351 strain of RHDV1, which were previously circulating in Australia (Mahar *et al.* 2018b; Peacock *et al.* 2017). RHDV2 has been shown to produce increased mortality in kittens (Neave *et al.* 2018), and at Turretfield have produced epizootics early in the breeding season, which may substantially affect future rabbit survival dynamics, reducing the importance of birth timing and increasing the importance of maternal RHDV2 antibodies, genetic resistance, or environmental factors.

The effectiveness of maternal myxomatosis antibodies in protecting offspring from the disease has so far been unclear. Fenner and Marshall (1954) found increased survival and signs of disease attenuation in juveniles with maternal antibodies when challenged with myxomatosis, whereas Sobey and Conolly (1975) found no effect. It appears that the virulence of the myxomatosis strain used, and its dosage, may determine the efficacy of maternal antibody protection. Our study is the first to assess the impact of maternal myxomatosis antibodies in wild rabbits and finds a significant increase in survival for the offspring of females that test positive for myxomatosis antibodies. This result supports Fenner and Marshall's conclusion regarding the protective nature of maternal myxomatosis antibodies and indicates that the effect is relevant under field as well as laboratory conditions. An alternative interpretation could be that the impact of myxomatosis antibodies is related to maternal age, as myxomatosis outbreaks occur in most years at Turretfield meaning that older dams are almost always survivors of the disease. However, the decrease in offspring survival that we observed associated with dam age implies that age does not drive the antibody effect.

Our finding of decreased offspring survival associated with maternal age (Table 1, Figure 4b) was unexpected, given that Rödel *et al.* (2009) found the opposite, although we did record low survival of offspring from dams in their first year (Figure 3c). The discrepancy between studies could be a result of pre-weaning differences, because all rabbits in our study were

trapped after emergence from the burrow, whereas Rödel *et al.* included nestlings in their survival calculations. Conceivably, old dams may have more experience in nurturing nestlings but produce less milk, resulting in high weaning success but weaned offspring that are smaller (as noted by Rödel *et al.* 2009) and have reduced subsequent survival.

Significance

Breeding and dispersal strategies have a profound effect on the distribution of alleles throughout populations (Chesser 1991a; Chesser 1991b; Parreira and Chikhi 2015; van Staaden 1995). Because allelic diversity is the substrate on which both natural selection and genetic drift can act, these strategies have long-term consequences on the ability of populations to adapt to new stressors (e.g., Caprio and Tabashnik 1992; Hughes and Boomsma 2004). The socially structured breeding strategy exhibited by rabbits, particularly in combination with sex-biased dispersal, creates an excess of heterozygosity within breeding groups. This excess heterozygosity reduces the effects of genetic drift and inbreeding, maximising the available allelic diversity for adaptation and may therefore aid the proliferation of innovations such as RHDV resistance. However, typically short dispersal distances will reduce the rate at which local genetic innovations spread at a wider scale. It therefore seems likely that genetic resistance to RHDV, which has been observed patchily across Australia (Elsworth *et al.* 2012), may have developed independently in different regions. This possibility will be important to keep in mind for any future studies that attempt to identify loci associated with RHDV resistance, because they may not be homologous across the landscape.

The use of population models to optimise management strategies for threatened or pest species is reliant on an accurate understanding of factors that influence recruitment. For example, Wells *et al.* (2016) refined a previous analysis by Fordham *et al.* (2012) by incorporating age-structured seasonal survival rates into their simulation models to guide development of rabbit management regimes. This additional demographic information revealed the importance of targeting reproductive age classes in order to achieve population extirpation. Based on our findings, these models could be refined even further by incorporating myxomatosis antibody status and birth month as factors influencing juvenile mortality rates.

The increased offspring survival in large warrens during this study warrants further investigation under targeted experimental conditions. After finding that rabbits occupy larger warrens at lower density, and with no greater reproductive output, Rouco *et al.* (2011) recommended using smaller artificial warrens for restocking in rabbit conservation programs. If the effect of warren size on offspring survival is replicated in artificial warrens, then this recommendation should be reconsidered in order to maximise population growth outcomes.

In this study we have shown multiple paternity and a high prevalence of extra-warren breeding in wild rabbits for the first time. We suggest that, while still important, the social structure of breeding in rabbits may be less strict than previously thought. We have also demonstrated the impact of larger warrens, maternal myxomatosis antibodies and birth month in determining offspring survival. Considering these factors when designing rabbit management strategies, for example by baiting pest rabbits with RHDV outside of the breeding season to avoid immunising resistant kittens, will create optimal outcomes for both ecosystems and agriculture.

Chapter 5 – Coevolution of rabbits and RHDV in Australia

As with myxomatosis, the coevolution of rabbits and RHDV has profound ecological and economic ramifications through its impact on rabbit abundance in their native and invasive ranges. The aspiration of my research outlined in previous chapters was, with a sufficiently thorough understanding of the processes involved, we will be able to perturb coevolutionary dynamics to better manage rabbit populations in Australia and internationally. In this chapter I examine the implications of my combined research findings for rabbit-RHDV coevolution, rabbit management practices and the impact of new RHDV strains on Australian rabbit populations. I examine these areas with particular attention to the limitations of current understanding, where future research can be directed to achieve our aspiration of improved rabbit management through viral biocontrols.

A molecular arms race

Coevolution of viruses and their hosts is generally thought to be driven by a molecular arms race between host antiviral mechanisms and viral counter-mechanisms (e.g., Marques and Carthew 2007). This arms race was introduced by Van Valen in 1974 as the Red Queen's Hypothesis, named for Lewis Carroll's character who famously declared that "it takes all the running you can do, to keep in the same place". Under this process, when either species develops a beneficial mutation it disadvantages the other, increasing selective pressure and thus producing continuous genetic innovation without either species necessarily outcompeting the other. In this manner, viral resistance is generally assumed to be a major driver of rabbit and RHDV evolution.

Rabbit resistance and RHDV virulence have been previously studied by Elsworth *et al.* (2012, 2014) using viral challenge experiments. At Turretfield in South Australia, where rabbits were shown to be resistant to RHDV, Elsworth *et al.* (2014) found that field strains of RHDV increased in virulence over a period of three years, as predicted by the Red Queen's Hypothesis. They also found increased resistance in wild rabbits, as compared to commercial domestic rabbits, which had not had an opportunity to adapt to RHDV, although this effect varied throughout the country (Elsworth *et al.* 2012). Adaptation to new stressors, such as RHDV, depends on both the underlying genetic variability of a population upon which natural selection can act, and rates of gene flow to spread beneficial mutations. To examine how these parameters may contribute to the observed distribution of RHDV resistance I studied the influences of rabbit genetic structure at both landscape (Chapter 3) and local scales (Chapter 4). My findings indicated that the landscape scale variability of Australian rabbits is dominated by six ancestral clusters representing historical introduction bottlenecks. At a local population scale the rabbit's socially structured polygynandrous breeding strategy maximises heterozygosity, facilitating rapid adaptation. However, very limited dispersal distances are likely to restrict the rate of geneflow at larger scales.

In light of these results, I interpret the patchy RHDV resistance observed by Elsworth *et al.* (2012) as probable indication of multiple independent adaptations. Given the geographic variability in neutral genetic loci observed in Chapter 3, it is entirely possible that these independent adaptations each reflect unique mutations or collections of mutations. If this is the case, rabbits in different regions may have different strain-specific resistances analogous to the resistance of different human blood groups toward different strains of Norovirus (reviewed in Le Pendu *et al.* 2006). To understand the relationship between variations in RHDV resistance phenotype and landscape genetics, future studies will need to identify specific resistance alleles and their mechanisms, and to characterise them across populations. If these alleles differ at a landscape scale, then a simple genome-wide association study (GWAS) approach may not be successful. I would recommend partitioning genetic data into groups based on the ancestral clusters identified in Chapter 3 prior to searching for alleles associated with RHDV resistance.

While the clusters identified in Chapter 3 provide a useful starting point for understanding the genetic structure of Australia's rabbits, my study was limited by the use of volunteer hunters to collect samples. Sparsely inhabited areas of Australia were not well represented in my dataset, creating ambiguity about the locations of cluster boundaries and the true extent of the smaller three clusters. Extending this study with additional, targeted, sample sites would reduce these ambiguities, so that ancestral genetic groups can be more accurately compared in further studies. Inclusion of potential source sites from Europe would further clarify the impact of introduction history on the current genetic structure of Australian rabbits.

In the molecular arms race described at the beginning of this chapter, RHDV is advantaged by its rapid rate of evolution (Kovaliski *et al.* 2014), which is characteristic of RNA viruses that have short generation times and less stable genetic sequence (Domingo and Holland 1997). Recombination between RHDV variants provides an additional source of variability (Forrester *et al.* 2008; Lopes *et al.* 2015) and was particularly common in my study (Chapter 2); perhaps because of my intensive local sampling strategy. I found that variation in RHDV was not temporally separated within years. Multiple clades co-circulated in flies, of which a single variant was responsible for all recorded carcasses at any given site. This finding suggests that competition between variants for rabbit hosts creates a substantial selective pressure towards increasing infectivity. The first variant to infect a rabbit in a local population is likely to trigger an outbreak as that rabbit's carcass becomes a lucrative source for further transmission both within and between populations (Henning *et al.* 2005; McColl *et al.* 2002b; Mitro and Krauss 1993). This drive for increasing infectivity may explain the dominance of RHDV2 which has largely replaced the original RHDV1 strains worldwide (Calvete *et al.* 2014; Le Gall-Reculé *et al.* 2013; Lopes *et al.* 2014). In the absence of widespread RHDV1 outbreaks since the emergence of RHDV2, variant monitoring using fly traps as outlined in Chapter 2 will reveal how long RHDV1 continues to circulate at all.

RHDV2 has been shown to overcome both RHDV1 antibodies (Peacock *et al.* 2017) and innate kitten resistance (Neave *et al.* 2018; Neimanis *et al.* 2018). Increased infectivity towards a previously resistant portion of the population likely improves its chances of causing the first infection in each season, giving RHDV2 a competitive edge over RHDV1. I hypothesise that RHDV2 will negate the 'birthday effect' observed in Chapter 4 whereby rabbits born in Aug and Sept have dramatically higher survival rates, probably due to their innate immunity when exposed to an annual RHDV1 outbreak before 12 weeks of age. Testing this hypothesis will require the continuation of the Turretfield capture-mark-recapture project. Long running studies, such as Turretfield, are particularly valuable for investigating before and after effects of unexpected events like the arrival of RHDV2 in Australia. Although infectivity appears to be a critical attribute under selection for RHDV, the importance of necrophagic blowfly vectors for transmission between rabbit populations suggests that substantial decreases in the mortality rate of infected animals are unlikely. The 'Red Queen' should favour equilibrium in this respect.

Myxomatosis is commonly cited as a model for host-pathogen coevolution because its introduction to Australia in 1950 created an effective grand scale evolutionary experiment. The changes to both host and virus, leading to attenuation and reduced mortality, have been well studied since (as reviewed by Kerr 2012). The differing vectors for transmission (biting insects versus necrophagic flies) fundamentally shift the evolutionary dynamic of myxomatosis and RHDV, highlighting the importance of post-death transmission for RHDV. The significance of post-death transmission is often overlooked, but is extremely important to consider; as demonstrated by Weitz and Dushoff (2015) when considering the spread of Ebola in humans. As we learn more about the interactions between rabbits and RHDV this system is becoming an increasingly valuable model of coevolution to contrast with myxomatosis.

Rabbit management

As well as contributing to theoretical understanding of rabbit-RHDV coevolution, my studies make practical advances, which can be applied in the field for viral monitoring and rabbit management. The wind-oriented fly traps piloted in Chapter 2 were effective for catching flies and were shown to be more effective than carcass searches for detecting the presence of RHDV. Because these traps are inexpensive and require minimal time or skill to operate, they will be ideal for implementation in landscape-scale viral monitoring programs. These programs can be used to monitor the spread of RHDV around Australia and test the effectiveness of newly introduced strains such as K5. Additionally, programs like this could monitor for incursions of other fly-borne diseases of concern (e.g., avian influenza (Sawabe *et al.* 2006) and Newcastle disease virus (Barin *et al.* 2010)), and provide an early warning system for potential outbreaks. Vector-based monitoring is particularly powerful when combined with next-generation sequencing techniques which can identify specific variants

from within a sample. This gives us the potential to track outbreaks over space and time, identify their sources and observe their evolution.

In Chapter 4 I reported a significant increase in kitten survival with increasing warren size. Current recommendations promote small structures for artificial warrens used in rabbit re-stocking on the Iberian Peninsula, based on rabbit density and the reproductive output parameters estimated by Rouco *et al.* (2011). Given my findings, these recommendations may need to be revised to maximise population growth outcomes. However, an explicit controlled study is needed to validate whether this effect applies in different ecosystems and with artificial warrens. It is possible that large warrens are not the cause of increased survival, but a result of high-quality landscape patches that benefit survival and support larger populations.

Pest management Decision Support Systems (DSS) are designed to assist public or private landholders with optimising strategies for pest control. For a given set of input parameters (e.g., land size, climate data, current pest population) they assess whether pest control activities are needed to achieve economic or environmental outcomes, and if so, what the most appropriate control strategy would be. The more sophisticated DSS, such as *EntomoLOGIC* used to manage insect pests in the cotton industry (Bange *et al.* 2004), simulate future pest abundance using internal population models. These models rely on knowing which are the parameters that drive population dynamics and having accurate estimates of those parameters. While several DSS for rabbits exist (McGlinchy 2011; PestSmart 2017), these have seen limited uptake to date and are constrained by incomplete knowledge of local parameters. In Chapter 4 I contributed to the knowledge base informing rabbit management DSS by identifying parameters contributing to offspring survival. My results indicate that warren size is a relevant, previously overlooked parameter to include in DSS when estimating survivorship. When considering the seasonality of breeding, models should also include the more restricted seasonality of offspring survivorship shown in Chapter 4. I also contribute to the body of knowledge documenting dispersal rates in rabbits, a parameter which appears to vary substantially between sites (Parer 1982; Richardson *et al.* 2002). What causes this variation remains unclear and would be an interesting avenue for further research because dispersal affects the rate at which beneficial genetic mutations can spread and the rate at which rabbits can re-invade following local control efforts. Understanding the dispersal parameters of the rabbit is thus an important step in optimising rabbit management strategies.

Previous understanding of rabbit dispersal has been characterised by a disconnect between the short distance of documented dispersals in warren-based studies (Künkele and von Holst 1996; Parer 1982; Richardson *et al.* 2002) and the rapid invasion of rabbits across Australia between 1860 and 1910. This range expansion has been described as the world's fastest

mammal invasion (Caughley 1977), and was immortalised in Australia's social narrative by accounts such as the following:

> *"…the rabbits had come in such millions that the whole ground seemed to move…The two of them might as well have tried to sweep back the Sahara with the broom, they reckoned, as try and stem that army." (Ratcliffe 1938)*

My findings in Chapters 3 and 4 supported the conventional ecology of limited dispersal in rabbits. In chapter three I show that Australia's rabbits are not panmictic. At least six genetic clusters were detected, indicating that geneflow has not been sufficient to overcome the differentiating effects of multiple introductions and genetic drift in over 100 generations. In Chapter 4 I found that average dispersal distances in juvenile rabbits are less than 200m to neighbouring pre-established warrens, supporting the findings of other studies (Künkele and von Holst 1996; Parer 1982; Richardson *et al.* 2002). Taking these results together I tentatively conclude that reinvasion of rabbits into locally eradicated areas should be low, especially if all warrens are destroyed to remove harbours for short-range dispersal along the boundaries. Ideally, future experiments would explicitly measure re-invasion across various distances, both with and without warren destruction, to enable the longevity of rabbit extirpation projects to be estimated.

How, then, can we decipher the discrepancy between ecological and historical wisdom? Two hypotheses present themselves: either rabbit behaviour is considerably altered at an invasion front (which has been observed previously in cane toads (Alford *et al.* 2009; Gruber *et al.* 2017)); or the low rate of long-distance dispersal was still adequate to create rapid regional range expansions capitalising on the human-mediated dispersal evidenced in Chapter 3. The former hypothesis could be facilitated by extreme density gradients between invading rabbit populations experiencing low predation pressure and unoccupied lands, and by reduced social barriers to dispersal in virgin habitat. Alternatively, based on the findings of Richardson *et al.* (2002), low rabbit density on the invasion front may encourage greater dispersal distances, thereby increasing the pool of available mates to maintain an adequate effective population size. The hypothesis that infrequent long-range dispersals as presently observed are sufficient to explain rapid range expansions can be explicitly tested using computer simulations, but these would need to be informed by accurate dispersal range distributions. All population-based studies of rabbit dispersal to date, including ours in Chapter 4, have been limited by the size of the sampled warren area and may therefore underestimate the frequency of long-range dispersal. Radio tracking experiments (e.g., Jennings and Mutze 2017; Moseby *et al.* 2005) are not as limited in geographic scale, but small sample sizes to date have reduced the chance of detecting the few individuals that become long distance dispersers. As GPS technology becomes increasingly miniaturised and

affordable, a larger-scale GPS tracking experiment (with n > 100) targeting juvenile rabbits would be ideal for determining the true extent of modern long-range dispersals.

The future of RHDV in Australia

Field sampling for all three of the studies presented in this thesis was conducted in 2013 and 2014, when all known RHDV strains in South Australia were descendants of the originally introduced Czech-351 strain of RHDV1. Since this time, three new strains have begun circulating in Australia. In 2014 a recombinant strain of RHDVa was detected near Sydney where it persists locally (Mahar *et al.* 2018b). RHDV2 was first detected in Australia in May 2015, and within 18 months had spread throughout the country and begun replacing the original RHDV1 strain (Mahar *et al.* 2018a; Mutze *et al.* 2018). In March 2017 a new Korean RHDVa variant known as 'K5' was intentionally introduced throughout Australia as a novel biocontrol agent (World Organisation of Animal Health 2017). While initial reports indicate that K5 has been effective at producing localised outbreaks (Adams 2017), whether it will establish and circulate independently will be determined by its ability to compete with the existing RHDV1 and RHDV2 strains.

To monitor the new epidemiological environment during this period of transition I recommend wide-scale implementation of fly-traps, as piloted in Chapter 2. Because these traps are so simple to use and cost-effective, they could be run by community 'citizen scientists' such as school groups, who would send their flies to a laboratory for diagnostic testing. This landscape-scale monitoring would reveal:

- Any incursions of novel RHDV strains
- Whether the original Czech-351 derived strain is still circulating in Australia
- Whether K5 persists and circulates independently of baiting programs, and if so, how far it has spread
- Whether variability of RHDV at a national level is comparable to that observed regionally in our Chapter 2
- Whether the most competitive RHDV strains originate in specific regions
- Whether patterns of RHDV dispersal are predictable based on temperature or wind patterns

RHDV2 is known to be capable of infecting rabbits with antibodies to RHDV1 (Dalton *et al.* 2014; Peacock *et al.* 2017); if the inverse is also true then monitoring may reveal alternating dominance of the two serotypes as increasing population immunity to one advantages the other. However, evidence from overseas where RHDV2 has been circulating since 2012 suggests that RHDV2 completely replaces RHDV1 (Calvete *et al.* 2014; Le Gall-Reculé *et al.* 2013; Lopes *et al.* 2014). Based on my finding that multiple RHDV strains circulate concurrently, in direct competition for rabbit hosts (Chapter 2), it appears likely that RHDV2's ability to outcompete RHDV1 stems from its increased infectivity towards juvenile rabbits (Dalton *et al.* 2014; Neave *et al.* 2018; Neimanis *et al.* 2018).

The infectivity of RHDV2 towards young rabbits is likely to dramatically change the established RHDV outbreak dynamics in Australia. My results in Chapter 4 indicate that, prior to RHDV2, the timing of kitten births had a substantial impact on survival, correlating with the age of first exposure to RHDV1. Under a system dominated by RHDV2, I expect the age of exposure to be less important, while maternal antibodies may have a more substantial impact. Without kitten immunity delaying the susceptibility of new recruits each year, I would expect RHDV2 to produce outbreaks earlier in the breeding season. In the three years since RHDV2 was first detected at Turretfield we have indeed observed a change in outbreak timing towards the beginning of the breeding season, as well as a marked population crash which may result from the combined effects of RHDV2 and myxomatosis. An initial crash is to be expected when a new serotype infects a naïve population; whether the population remains depressed in the long term remains to be seen.

Currently, we know that RHDV2 can infect and kill young kittens due to incidental observations (e.g., Dalton *et al.* 2014; Dalton *et al.* 2012), and subsequent small pathology studies using domestic rabbits (Neave *et al.* 2018; Neimanis *et al.* 2018). To understand the likely epidemiological changes in response to RHDV2 and K5, a larger challenge study examining the infectivity and mortality rates of both strains in kittens, as well as mature rabbits, would be enlightening. To properly reflect a field situation, this study would need to involve wild-sourced rabbits because domestic rabbits have not had the opportunity to adapt to RHDV and are more easily infected (Elsworth *et al.* 2012). Due to the difficulty of trapping wild rabbits in sufficient quantity, a colony founded from wild-caught rabbits, from a resistant population, should be established to facilitate this kind of study in Australia. In the early stages of this project I attempted to establish such a colony from 13 sub-adult rabbits trapped at Turretfield and subsequently housed in commercial rabbitry facilities. Unfortunately, these rabbits bred poorly and failed to nurture their offspring; after 18 months the attempt was abandoned. I hypothesise that the rows of individual cages at a commercial rabbitry inhibit the formation of social structures necessary to reduce stress and promote breeding in wild rabbits (whereas commercial breeds have been selected for tolerance to these conditions). Future attempts to establish colonies using wild rabbits may benefit from larger, communal pens with adjoining private nesting areas to simulate breeding stops.

Conclusions

In this thesis I have taken advantage of modern next-generation sequencing techniques to examine genetic factors that contribute to coevolution between RHDV and the European rabbit. I examined the genetic variability in local RHDV, and in rabbits at both local and national scale, and updated rabbit breeding and recruitment parameters used for optimising management strategies.

Key developments contributed by this project include: i) validation of fly traps as a monitoring tool; ii) the discovery of six genetic clusters in Australian rabbits, reflecting at least three primary introductions; iii) detection of multiple paternity and frequent extra-warren breeding in wild rabbits; and iv) finding that kitten survival prior to the spread of RHDV2 was affected by birth timing, maternal myxomatosis antibodies and warren size, but not maternal RHDV antibodies.

My findings will enhance field monitoring techniques, empirical models of coevolution and strategies for rabbit management. These developments are a stepping stone that will facilitate future research activities to improve rabbit management as a pest in its invasive range and as a species of conservation concern in its native range in Europe.

Appendix 1: Reference list of RHDV strains in Australia

Based on the nomenclature guidelines for lagoviruses proposed by Le Pendu *et al.* (2017), all varieties of RHDV fall into the genogroup G1, within the species *Lagovirus europaeus*, from the family *Caliciviridae*. Below I provide the common name for each variant, followed by its designation according to Le Pendu *et al.* in brackets and a brief description of its introduction to Australia:

RHDV1 (G1.1):

> Czech-351 (G1.1c) - the first strain introduced to Australia. Escaped quarantine on Wardang Island (South Australia) in 1995 and quickly established recurrent epizootics throughout the country. All RHDV sequences examined in this thesis are descendants of this strain.
>
> RHDVa (G1.1a) - Two variants of RHDVa exist in Australia. The first is genetically similar to a strain in China and was detected in NSW in 2014 and has not been found beyond a limited area in NSW and the ACT (Mahar *et al.* 2018b). The second, known as 'K5', was sourced from Korea and introduced to Australia deliberately as a biocontrol in 2017 (World Organisation of Animal Health 2017)

RHDV2 (G1.2): First identified in Australia in 2015 with likely origins in southern Europe (Hall *et al.* 2015), this new serotype appears to be replacing RHDV1 throughout the world (Lopes *et al.* 2015; Mahar *et al.* 2018a)

RCV-A1 (G1.4): A benign serotype offering partial cross-protection from RHDV, which appears to have arrived in Australia prior to the other strains, possibly in the 1950s (Mahar *et al.* 2016).

Appendix 2: Supplementary tables for Chapter 2

Supplementary Table 1: RHDV capsid primer sequences as published by Kovaliski *et al.* (2014).

Primer name	Sequence (5'-3')	Genome position (bp)
RHDVF4846	CCgATgATggTgAgYCTYTTRCCTgC	4846
RHDVR6059	TgRCCgTTCCACCTgTTgTCATTgC	6059
RHDVF5926	gCAATYCAggTRACAgTggAAACAAggC	5926
RHDVR6986	CCAggTTgAACACgAgYgTgCTYTTgg	6986

Supplementary Table 2: Fly Catch binomial GLM model-averaged coefficients (full average). Reference site was Concordia and reference year was 2013. Significant terms at $\alpha = 0.05$ are marked with a *

	Estimate	Std. Error	Adjusted SE	z value	Pr(>|z|)
(Intercept)	5.042	0.292	0.298	16.899	< 0.0001*
Nights	0.067	0.020	0.020	3.315	0.001*
Site-Marananga	0.524	0.265	0.270	1.938	0.053
Site-Rosedale1	1.496	0.260	0.266	5.630	< 0.0001*
Site-Rosedale2	0.437	0.319	0.326	1.343	0.179
Site-Wasleys	1.194	0.327	0.334	3.571	0.000*
Year-2014	-0.414	0.246	0.249	1.665	0.096
Date	0.000	0.002	0.002	0.066	0.947

Supplementary Table 3: Number of unique contigs assembled by MIRA for each sample site and date. P indicates a positive RHDV1 PCR sample that did not produce useable sequence data. N indicates samples for which RHDV1 PCR was negative. X indicates unsampled sites/dates.

DATE	ROSEDALE 1	MARANANGA	CONCORDIA	ROSEDALE 2	WASLEYS
10/09/2013	15	P	N	X	X
17/09/2013	2	N	N	X	X
24/09/2013	16	N	4	X	X
28/09/2013	N	1	N	X	X
1/10/2013	6	1	6	X	X
8/10/2013	4	P	5	X	X
10/10/2013	5	1	X	X	X
15/10/2013	4	3	X	X	11
19/10/2013	P	X	4	X	X
1/11/2013	8	X	X	3	X
4/11/2013	13	4	N	X	6
15/11/2013	31	2	N	N	1
21/11/2013	2	N	10	N	N
28/08/2014	2	N	4	1	P
7/09/2014	52	7	X	7	5
10/09/2014	26	X	X	11	X
16/09/2014	30	2	X	1	3
24/09/2014	P	2	X	10	3
7/10/2014	X	1	N	X	X
15/10/2014	N	11	N	N	N

Supplementary Table 4: RHDV variant detection negative binomial GLM model-averaged coefficients (full average). Note that no non-recombinant RHDV sequences were detected at Rosedale2 in 2013, resulting in an enlarged standard error for this site.

2013 RHDV Outbreak					
	Estimate	**Std. Error**	**Adjusted SE**	**z value**	**Pr(>|z|)**
(Intercept)	-0.174	1.098	1.118	0.155	0.877
Site-Marananga	0.000	0.729	0.752	0.000	1.000
Site-Rosedale1	1.103	1.112	1.130	0.977	0.329
Site-Rosedale2	-11.750	1513.000	1561.000	0.008	0.994
Site-Wasleys	-0.409	0.994	1.021	0.400	0.689
Clade-2	-1.211	0.884	0.898	1.348	0.178
Date	0.008	0.016	0.016	0.506	0.613
2014 RHDV Outbreak					
	Estimate	**Std. Error**	**Adjusted SE**	**z value**	**Pr(>|z|)**
(Intercept)	-0.054	0.634	0.644	0.084	0.933
Date	-0.014	0.018	0.018	0.792	0.428
Clade-2	-0.066	0.382	0.391	0.168	0.867
Clade-1	0.177	0.471	0.478	0.370	0.711

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