DEVELOPMENT OF GENETIC RESISTANCE TO RABBIT HAEMORRHAGIC DISEASE IN WILD RABBITS *Oryctolagus cuniculus*

by

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The research undertaken in this thesis was covered by Animal Ethics Approval from the relevant state agencies. Rabbits were collected under approvals: Queensland Pest Animal Ethics Committee approval PAEC060601, South Australia Wildlife Ethics Committee approval WEC45/2007, PIRSA Animal Ethics Committee approval AEC09/03 and Victorian Animal Ethics Committee approval 062793. Experimental trials were conducted under approvals: Queensland Pest Animal Ethics Committee approvals PAEC060601 and PAEC060801 and Queensland Community Access Animal Ethics Committee approvals CA2007/10/220 and CA2009/06/356.

Publications associated with this thesis

This thesis includes publications for which I am the senior but not the sole author. I took the lead in this research in that I designed the research, undertook the experimental work, analysed the data and wrote the manuscripts. I was, however, assisted by my co-authors.

The publications associated with this thesis are as follows:

Chapter Three

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Chapter Four and Five

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Rabbit haemorrhagic disease virus (RHDV) was introduced into Australia in the mid 1990's to aid the control of rabbits. The impact was immediate and highly successful generally causing major declines in rabbit numbers. Some ten to fifteen years after the introduction of RHDV, increases in rabbit populations were seen, in some cases reaching pre-RHDV levels. This raised questions of the continuing efficacy of RHDV and whether rabbits had developed resistance. Resistance is a natural consequence of co-evolutionary processes and can reduce the impact of viral infection. This was the case as rabbits developed innate resistance against myxoma virus. The impact of myxoma virus was greatly reduced and rabbit populations recovered although not to the levels seen pre-myxomatosis.

To test for resistance in rabbits to RHDV, direct challenge experiments were used to compare different wild populations against each other and against unselected domestic controls. Susceptible rabbits from twelve populations throughout central and eastern Australia were used to represent a range of environmental habitats. In the absence of temporal information of resistance levels, a spatial experiment can be used to infer resistance as different populations are expected to evolve at different rates. The infection and mortality rates varied between the populations (from 0% infection at Ingliston to 73% infection at Bulloo Downs) tested demonstrating that resistance has developed against RHDV.

The establishment that resistance is developing in Australian rabbits raised further questions about the mechanism of resistance given the observed differences between populations. Of key importance is whether there is a genetic basis to the resistance

and whether the virus is evolving to keep pace with the resistance. Further challenge testing provided answers for these questions as did comparative information from molecular research from France.

Resistance levels appeared to be related to average annual rainfall from the sites with lower levels of resistance seen at arid sites and areas with high rainfall. It is likely that this pattern is caused by poor recruitment in arid areas and the presence of a partially protective non-pathogenic lagovirus in cooler-wetter sites. The mechanism of resistance appears to be one which prevents infection. A view that is supported by molecular research from France that suggests that the phenotype of ABH binding antigens influence survival against exposure to low doses of RHDV. Similarly, the challenge experiments presented in this thesis showed that wild rabbits were better able to survive challenge with low doses of RHDV compared to unselected domestic rabbits, but that this difference disappeared at high doses. Taken together, these findings suggest that resistance to a low dose simply delays infection until a large dose is encountered as most adult rabbits have antibodies to RHDV. As the selective advantage for rabbits in doing this is unclear, a dual mechanism of infection is postulated with resistance preventing a virus facilitated uptake which may allow rabbits to slow infection and perhaps better control it.

The phenotype for resistance may readily be achieved. Challenge tests in a laboratory situation were used to expose resistant individuals from a wild population with low resistance. Breeding from only these resistant individuals produced a fully resistant generation. This shows that resistance is heritable.

Finally, preliminary examination of field strains of RHDV indicates that virulence is being maintained at a high level given that recently collected field strains infected a higher proportion of test rabbits and caused greater mortality than the Czech strain virus originally released. The evidence that the virus is maintaining its virulence relative to rabbit resistance is encouraging but given that rabbit numbers are slowly increasing it may be possible that resistance is slowly out-stripping virulence. Evolution of RHDV and rabbits is a continuing process and the effectiveness of the virus in controlling rabbits may be a population specific prospect and other control tools such as warren ripping, poisoning and fumigation will be required at sites with high levels of resistance.

CHAPTER ONE

A GENERAL INTRODUCTION TO RABBITS AND CONTROL IN AUSTRALIA

Pre-Rabbit haemorrhagic disease

The introduced European rabbit (*Oryctolagus cuniculus*) has long been a major pest in Australia and still causes major economic losses. Rabbits were introduced in 1859 to provide hunting sport for colonists. In the late 1890's and early 1900's, large-scale fences were erected in an attempt to stop or slow the spread of rabbits. However they inevitably spread, establishing in a wide range of habitats from alpine to desert (Myers and Parker 1965). Recent impact statements on the cost of pest animals operate on a triple bottom line model that includes impacts on the economy (traditionally agriculture and management costs), as well as environment and social factors. Economic impacts can be directly estimated through production losses and control effort, but environmental and social costs are more difficult to evaluate.

Economically, rabbits are one of the worst vertebrate pest species in Australia and despite major advances using biological control agents and applied controls such as poisoning, warren ripping and fumigation, they currently cause a conservatively estimated $206 million in lost agricultural production and management annually (Gong et al. 2009). Rabbits compete with cattle and sheep for pasture, and eat horticultural crops. Based on sheep dry-weight equivalents, it has been estimated that 12 - 16 rabbits consume the same as one sheep (Short 1985; Mutze 1991). In the five years after the introduction of myxomatosis, wool, sheep and cattle production in

NSW all increased by around 25% (Waithman 1979). Damage to horticultural crops can be more severe as the rabbit does not need to consume the entire plant to make it unsaleable. The effectiveness of myxoma virus waned and by the early 1990's (prior to the introduction of RHDV) the annual cost of rabbits to wool, stock and crop production had risen to an estimated $600 million (ACIL 1996).

Environmentally, rabbits denude the landscape, prevent regeneration of vegetation and compete with native animals for burrows and food. Rabbit warrens provide a disturbed environment and altered soil composition which tends to promote exotic plants over native species (Eldridge and Simpson 2001). Rabbits alone can also eliminate *Acacia* seedlings and suppress the regeneration of seedlings in arid regions (Lange and Graham 1983; Cooke 1991). Similarly they alter the species composition of pastures and may have been responsible for the localised extinction of many species (Williams et al. 1995). Even at low densities of 3 rabbits per hectare, rabbits can maintain the dominance of exotic herbage over native species, but when rabbits are removed, native species return (Mallett and Cooke 1986). Rabbits are highly aggressive, being seen to attack even yellow-footed rock wallabies (*Petrogale xanthopus*) in enclosures (Poole et al. 1985). The decline of burrowing mammals has coincided with the arrival of rabbits (Williams et al. 1995) and now rufous hare-wallabies (*Lagorchestes hirsutus*) and the Bilby (*Macrotis lagotis*) are only found north of the rabbits distribution, or in sites where rabbits are rare, and the burrowing bettong (*Bettongia lesueur*) is no longer found on the mainland (Watts 1969; Southgate 1990; Williams et al. 1995). Rabbits also compete for forage with native animals. The diets of rabbits and yellow-footed rock wallabies become similar in drought conditions (Dawson and Ellis 1979) and spectacled-hare wallabies

(*Lagorchestes conspicillatis*) directly compete with rabbits for high nutritional, seasonally scarce green grasses (Ingleby and Westoby 1992). Even the range contraction of the common wombat (*Vombatus ursinus*) in South Australia may be due to competition with rabbits (Mallett and Cooke 1986).

Socially, rabbits had previously been considered of beneficial value in some circumstances. Rabbit harvesting was a significant industry before 1950 (Williams et al. 1995). The effect of myxomatosis reduced the number of wild rabbits forcing these industries to switch to using domestic rabbits (Williams et al. 1995). This greatly reduced the benefits of wild rabbits as an economic resource (Williams et al. 1995). The main negative impacts of rabbits on people are increased work for the landholder and the creation of warren structures. These reduce quality of the life as well as presenting hazards for livestock and horses, including those pulling ploughs and as a result many farmers ploughed around warrens (Fitzgerald and Wilkinson 2009).

Traditionally, the primary management of rabbits was conducted on a property by property basis. Land managers employed shooting, trapping, ferreting, poisoning, fumigation and destruction of warrens through ripping to control rabbits (Williams et al. 1995). Over time, the number of fences have been reduced or allowed to run into disrepair, such that only one fence, built to protect south-east Queensland and portions of the West Australian vermin fence remain.

The deliberate introduction in 1950 of the myxoma virus which causes the disease myxomatosis, had a dramatic effect on rabbit numbers in Australia, reducing the overall population by over 90% (Fenner and Ratcliffe 1965). This sudden and

widespread collapse in numbers provided a real opportunity for traditional control techniques to be improved and employed more strategically to reduce maintain rabbits at very low densities. However, resistance to myxomatosis developed within ten years and virus strains of myxoma virus were attenuating (Fenner and Fantini 1999) resulting in substantial increases in numbers. While these numbers did not reach levels seen before the release of myxoma virus, they did reach levels where they caused significant damage to environmental ecosystems and agricultural industries (Fenner and Fantini 1999). The release of European rabbit fleas (*Spilopsyllis cuniculi*) in the late 1960's helped the spread and persistence of myxoma virus in rabbit populations and altered the age structure of populations by reducing the number of young rabbits (Sobey and Conolly 1971; Shepherd and Edmonds 1978; Cooke 1983), although they failed to establish in regions where the rainfall is less than 200 mm per year (Cooke 1984). For this reason, the Spanish rabbit flea (*Xenopsylla cunicularis*), which is better suited to arid conditions was introduced in 1993 (Williams et al. 1995). This increased the efficacy of myxomatosis in arid regions and caused declines in these populations (Cooke 1995).

Post-Rabbit haemorrhagic disease

The discovery and subsequent introduction of rabbit haemorrhagic disease virus (RHDV) in 1995 again caused a great reduction in rabbit numbers across Australia (Fenner and Fantini 1999). At the time of its release, extensive monitoring was undertaken to assess the efficacy of RHDV. Similar to the myxomatosis situation, land managers were urged to use follow-up control techniques to take advantage of the low numbers of rabbits caused by rabbit haemorrhagic disease (RHD – the disease

caused by RHDV). Rabbit warren ripping has been identified as causing a long-term reduction in the number of rabbits and having more impact than just the presence of RHDV alone (McPhee and Butler 2010; Berman et al. 2011).

Where monitoring has continued, some sites have shown increases in rabbit numbers back to pre-RHDV levels (Sandell 2006; McPhee and Butler 2010). This is supported by unpublished information (e.g. M. Ridge, DDMRB, unpublished) from land managers and industry bodies who believe that rabbit impacts on their land management objectives and profitability are increasing (Australian Wool Innovations, unpublished). A similar pattern of increased rabbit numbers and damage was seen as resistance to myxomatosis and attenuated strains of myxoma virus appeared although rabbit number increases did not return to pre-myxomatosis levels (Fenner and Fantini 1999). In a co-evolutionary system between a virus and host, resistance development is a natural consequence of selective pressures forced by the virus (Woolhouse et al. 2002). It is to be expected that resistance against RHDV will develop in rabbits and this may be the cause of increases seen in some populations.

In this thesis the primary aim is to determine the continuing effectiveness of Rabbit Haemorrhagic Disease as a control agent in the management of wild rabbits in Australia. Evolutionary theory provides expectations that resistance in the host may develop and how virulence in the virus may change. Understanding these two processes will provide the information required to determine the role RHDV will play in the future management of rabbits. The objectives were, firstly to determine whether or not resistance to RHD was developing and secondly to attempt to find support for a genetic basis to resistance.

One of the major constraints in understanding co-evolutionary systems is the long time frames required to effectively measure changes in infection and disease responses (Woolhouse et al. 2002). The myxoma virus – rabbit system in Australia is one of the best examples where laboratory and field experiments performed after the introduction of the virus provided information on how resistance was developing and how the virus was evolving (Fenner and Fantini 1999). Unfortunately experiments to monitor resistance and virulence since the introduction of RHDV have not been done. Using the myxoma virus system and aspects of co-evolutionary theory, a framework of challenge testing to answer questions about RHDV can be developed.

Finally, an examination is conducted to determine if RHDV in the field is evolving. Again co-evolutionary theory provides directions for what may be expected. The trade-off theory of virulence evolution incorporates epidemiological factors of virulence and transmission of the virus as well aspects of host responses to suggest how virulence should evolve (Anderson and May 1982; Alizon et al. 2008). This model describes that where transmission of virus and virulence are linked in a positive manner, then virulence should increase, whereas if they are linked in a negative manner, there will be a trade-off and strains of virus with intermediate levels of virulence will be selected (Alizon et al. 2008). Strains of myxoma virus with intermediate levels of virulence became dominant in the field as a result of a compromise between transmission and virulence (Fenner et al. 1956), lending weight to some aspects of the trade-off theory of virulence evolution (May and Anderson 1983). The outcomes of virulence testing of RHDV may also aid in understanding current theory of evolution.

CHAPTER TWO

LITERATURE REVIEW OF RABBITS IN AUSTRALIA AND RESISTANCE AND VIRULENCE EVOLUTION

History of Rabbits and Control in Australia

European rabbits (*Oryctolagus cuniculus*) were successfully introduced into Australia, at Winchelsea in southern Victoria, in 1859. At the time, the introduction was primarily to provide sport through hunting for the landholders, mainly arrivals from Britain. Thomas Austin, a member of the Acclimatisation Society of Victoria and the man who had the initial 24 rabbits brought to Australia stated:

> *"The introduction of a few rabbits could do little harm and might provide a touch of home, in addition to a spot of hunting."*

It did not take long for the error of this judgement to be seen. Rabbits spread naturally, and as the interior of Australia was settled, rabbits were also carried and released to provide a food source for those following behind. The excellent conditions, lack of natural predators and these deliberate releases led to a rapid increase in rabbit numbers. Within 10 years, the original 24 brought over from England had risen to a level where 2 million could be shot in a year without impacting on their numbers (Rolls 1969). The rate of spread of rabbits in Australia was the fastest by any colonising animal anywhere in the world (Caughley 1977). Within 15 years rabbits had reached the New South Wales border (Rolls 1969; Stodart and Parer 1988), within 30 years the Queensland border and they reached the Western Australian and Northern Territory borders by 1900 (Williams et al. 1995).

It did not take long for the negative impacts of rabbits to be seen. They denuded the landscape, removed pasture for livestock and native animals and their digging caused erosion. Initial control practices involved everything from shooting to chemical fumigation. In 1886 fences were built in South Australia and Queensland and in 1901 in Western Australia, in an attempt to halt the spread. However, it was not until the introduction of biological control agents that rabbit numbers were reduced on a large scale.

Myxoma virus was introduced into Australia in 1950 and immediately reduced rabbit numbers. Initial declines of up to 99 per cent were seen across the country (Fenner and Ratcliffe 1965). Poisoning with strychnine and subsequently sodium fluoroacetate (1080) and later pindone was used to control rabbits as numbers increased and integrated control using several techniques was undertaken. The introduction of the European rabbit flea (*Spilopsyllus cuniculus*) in the 1970's enhanced the spread of myxoma virus (Sobey and Conolly 1971) especially into arid areas and helped keep rabbit numbers below the former extremes. Spanish rabbit fleas (*Xenopsylla cunicularis*) were introduced in 1990 (Cooke 1990; Mutze et al. 1998) when it was found that European rabbit fleas did not spread into arid areas. However, it was not until the introduction of Rabbit Haemorrhagic Disease Virus in 1995 that real headway was again made in reducing rabbit populations.

Myxomatosis

In its original hosts (*Sylvilagus braziliensis* and *S. californicus*) myxoma virus causes a persistent, benign infection with minimal cellular immune recognition and few clinical signs (Fenner and Ratcliffe 1965; Cameron et al. 1999; Nash et al. 1999). In the European rabbit, however, it causes a lethal disease. Following its discovery there was a push to examine the possibility of using it as a control agent for wild rabbits in Australia. After much debate and laboratory testing, the 'standard laboratory' strain of myxoma virus was introduced into wild rabbit populations in Australia in 1950 (Fenner and Ratcliffe 1965). The impact was dramatic causing declines of 99% in some rabbit populations (Fenner et al. 1953).

The success of the use of myxoma virus in Australia led quickly to similar releases and spread in Europe where it established over the entire continent (Fenner and Ratcliffe 1965). The Lausanne strain was released in Europe and produces a more florid disease with large protuberant skin lesions than the standard laboratory strain (Fenner and Ratcliffe 1965). The virus spread quickly reaching Spain, Holland, Belgium and Germany within 15 months of the original release in France in 1952 (Fenner and Chapple 1965; Fenner and Ratcliffe 1965). Myxoma virus caused a similar dramatic decrease in numbers in Europe as was seen in Australia and by 1953 in France and 1954 in Britain, laws were passed to attempt to halt the spread and outlaw deliberate releases of the virus (Fenner and Chapple 1965; Fenner and Ratcliffe 1965).

Selection pressures were great on both the rabbits and virus and within a few years resistance was developing in the rabbits and attenuated strains of the virus were being seen in Australia (Fenner and Marshall 1957; Marshall and Fenner 1958). A similar pattern emerged in Europe although the appearance of attenuated strains took longer (Fenner and Chapple 1965). It was thought this could be caused by the higher virulence of the originally released Lausanne strain in Europe in 1952 (Fenner et al. 1957).

Over time, the more virulent Grade I strains were rarely seen and the attenuated strains, including the low virulent Grade V strains that caused a < 50% case mortality rate, were dominant (Marshall and Fenner 1960; Fenner and Chapple 1965). As resistance continued to increase in rabbit populations, myxoma virus virulence has subsequently increased to keep pace with the rabbit resistance so that Grade III strains are dominant and Grade V strains are very rarely seen (Edmonds et al. 1975).

In wild rabbits myxomatosis now develops more slowly than in unselected domestic rabbits (Best and Kerr 2000). Survival time has not only increased, but disease severity is decreased as well (Best and Kerr 2000). This indicates that resistance slows the development of the disease and allows the rabbit to better control the infection. Myxoma virus, being a large DNA virus has incorporated host genes into its genetic makeup that allow it to regulate the rabbit's immune response (Upton et al. 1992; McFadden et al. 1995; Macen et al. 1996), also increasing the disease course, to increase the opportunity for replication and transmission. Replication of the virus and viral load in skin lesions on resistant rabbits is also decreased (Best and Kerr 2000) which would lead to decreased transmission. Despite the levels of resistance seen in

wild rabbits, death occurs in about 40 – 60 percent of rabbits that become infected (Parer et al. 1994; Best and Kerr 2000)

Rabbit Haemorrhagic Disease Virus

A new viral haemorrhagic disease was seen in domestic rabbits in the People's Republic of China in 1984 (Liu et al. 1984; Xu 1991) and was soon seen in the Republic of Korea (Park et al. 1991). In 1986 it appeared in domestic rabbits in Italy (Cancellotti and Renzi 1991) and quickly spread to other European countries (Morisse et al. 1991). It was first reported in wild rabbits in Europe in Spain in 1988 where it caused high mortalities, and within 5 years was maintaining rabbit populations at about 50% of their previous levels (Cooke 2002). Research into this new disease was rapid because of the commercial value of the rabbits in Europe (Cooke 2002) and the causative agent of the disease was established to be a calicivirus specific to the European rabbit (Ohlinger et al. 1990). Rabbit Haemorrhagic Disease Virus (RHDV), as it became known, was classified to the *Caliciviridae* family and subsequently to the *Lagovirus* genus (Green et al. 2000).

In common with all other caliciviruses, RHDV is a non-enveloped, positive-sense, single-stranded RNA virus (Ohlinger et al. 1990; Parra and Prieto 1990; Valíček et al. 1990; Figure 1). The genome contains 7437 nucleotides excluding the poly (A) tail arranged within two open reading frames (ORFs) (Meyers et al. 1991; Rasschaert et al. 1994; Gould et al. 1997). Wirblich et al. (1996) showed that within the two ORFs of the virus genome the larger ORF1 codes for several proteins including a

polymerase and the large VP60 as follows: NH2-p16-p23-p37- (helicase) - p30-VPg-TCP-(polymerase)-VP60-COOH. Proteins of uncertain function are indicated by the notation p followed by the number of amino acids they contain. Immunoblot analyses show that a minor structural protein of 10 kDa is encoded in ORF2 (Wirblich et al. 1996). König et al. (1998) infected cultivated rabbit hepatocytes with RHDV and demonstrated that 13 specific polypeptides were produced by the virus, the larger polyprotein producing smaller functional units on its severance by the viral protease. The large structural protein, VP60, is produced both by mRNA (subgenomic or sgRNA) and as part of the large polyprotein (Sibilia et al. 1995).

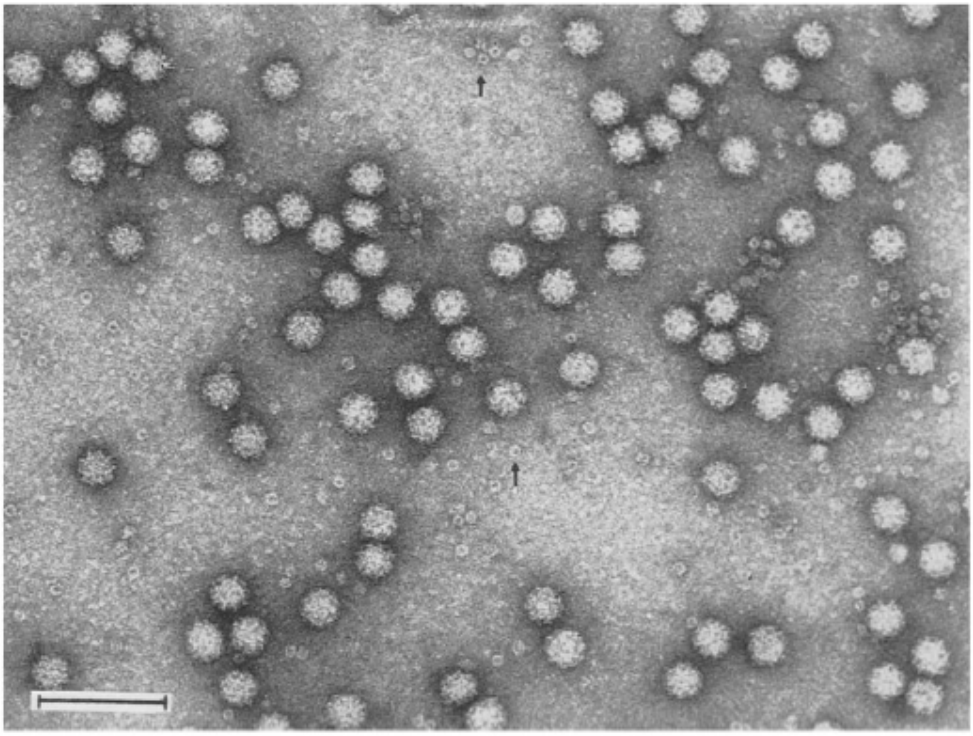

Figure 2.1. Electron-microscopic image of RHDV showing virions and ferritin-like structures (arrow). Bar = 100nm, from Valícek et al. (1990).

Rabbit haemorrhagic disease (the disease caused by RHDV) causes acute liver disease characterised by necrosis of hepatocytes (Park et al. 1995) and death is associated

with disseminated intravascular coagulation caused by liver break-down products (Xu and Chen 1989). Death occurs on average between 1 and 3 days after infection (Lenghaus et al. 1994; Westbury 1996; Cooke 2002). Survival times were initially shorter for wild rabbits which died more rapidly than domestic rabbits often with few outward signs of distress (Lenghaus et al. 1994). Infected rabbits become pyretic although body temperature may fall below normal in late stages of the disease (Marcato et al. 1991). Rabbits appear to behave normally and show few outward signs of disease (Cooke 2002). Bloody mucous discharge from the nose reported in domestic rabbits (Lenghaus et al. 1994; Cooke 2002) has not been reported in wild rabbits in Australia. Internally, the liver is discoloured with a reticulate pattern, the spleen is swollen and there may be haemorrhagic lesions in the trachea, lungs and occasionally the kidneys (Cooke and Fenner 2002).

Young rabbits (less than 12 weeks old) are less affected by RHDV having lower mortality rates and fewer signs of disease (Xu and Chen 1989; Marcato et al. 1991; Morisse et al. 1991). Young rabbits still develop hepatic lesions but these are not severe enough to lead to clinical disease or death (Mikami et al. 1999). This may be because macrophage and lymphocyte functions of young rabbits are intact but down-regulated, making them less likely to take up foreign particles when rabbits are under 4 weeks of age (Tomai et al. 1992). Kittens are also protected by maternal antibodies which affect the rate of disease and death with 90% surviving at 4 weeks, 50% by 6 weeks, decreasing to 10% by 9 weeks (Robinson et al. 2002b). This is an important consideration when selecting animals for experiments. Young rabbits can be chosen from the field as even if they have been exposed to RHDV they will not have developed disease or antibodies against disease, but they need to be raised to more

than 12 weeks of age before trials to ensure they are fully susceptible (Robinson et al. 2002b).

The impact of RHDV on wild rabbit populations in Europe suggested that it might be a useful tool for controlling wild rabbits in Australia. After an extensive search, a virus strain (Czech V351) from Czechoslovakia was chosen (Lenghaus et al. 1994) and testing commenced to ensure its efficacy, humaneness and species specificity (see Fenner and Fantini 1999; Cooke and Fenner 2002, for detailed reviews). During testing of the virus in a quarantine enclosure on Wardang Island in the Spencer Gulf off the coast of South Australia in 1995, the virus escaped and subsequently spread to the mainland (see Fenner and Fantini 1999, for a detailed review). This was a consequence of virus being transported off the island by insect vectors such as blowflies (Cooke 2002). Although attempts were at first made to contain this spread, the appearance of the virus over large areas of inland South Australia meant that further thoughts of a controlled but widespread release were abandoned. Nonetheless, it took a further year to officially register the virus and rabbits as agent and target organism under the *Biological Control Act (1984)* so that further virus releases in wild rabbits could be made. In the meantime the virus had spread naturally to all states and territories including Tasmania.

The initial impact of RHDV on rabbit populations was dramatic. RHDV spread quickly, travelling up to nine km per month in winter and 414 km per month in spring (Kovaliski 1998). In some populations, declines of up to 95 percent were seen (Bowen and Read 1998; Mutze et al. 1998; Bruce et al. 2004). However, some populations,

notably in regions of higher rainfall, did not experience as dramatic a decline in rabbit numbers (Saunders et al. 1999; Richardson et al. 2007). Indeed, in some of these areas, RHDV did not establish well, despite repeated efforts to introduce it (Bruce and Twigg 2005; Richardson et al. 2007).

A similar situation was seen in New Zealand. Rabbits were introduced in the mid 1800's for hunting and food for sailors and were reaching plague proportions in some areas by the end of the 1800's (Henning et al. 2005). They caused similar agricultural problems as those seen in Australia, but perhaps the biggest ecological impact they had was the decision by authorities to introduce mustelids and cats to aid in their control (Lough 2009). An attempt was made to introduce myxoma virus in the 1950's which failed due to a lack of vectors (Lough 2009). When Australia began looking into the viability of RHDV for controlling rabbits, New Zealand authorities assisted financially with the view of using that research to determine if the virus would be suitable for use in New Zealand. When the virus escaped onto the mainland of Australia, their interests ceased, however an application was made by landholders in New Zealand to import RHDV for rabbit control, but this was rejected (O'Hara 2006). Within two months of this decision dead rabbits were found on the South Island that were shown to have died of RHD (Thompson and Clark 1997) following the illegal introduction of RHDV (O'Hara 2006) and the virus quickly spread (Parkes et al. 2002). Coordinated releases were begun on the North Island soon after, but as with the Australian situation, the virus did not control rabbits as effectively in some populations (Parkes et al. 2002). Recently, some populations are beginning to increase in number which may be a result of RHDV becoming less effective, or the development of resistance (Parkes et al. 2008).

Recent evidence (Sandell 2006; McPhee and Butler 2010) shows that rabbit numbers in Australia are again increasing and in some cases to levels not seen since the introduction of RHDV. This raises the possibility that rabbits in Australia may be developing resistance to RHDV, much as occurred following the use of myxomatosis in the 1960's. Consequently, the wild rabbit in Australia provides the opportunity for the exploration into the relationships and interactions between virus and host. In this one host species, there are two pathological viruses (myxoma virus and RHDV) that have been deliberately introduced as biocontrol agents, and one of those has a non-pathogenic relative (RCV-A1) that was already circulating in rabbit populations.

Non-pathogenic rabbit caliciviruses

Following the discovery of RHDV in rabbit populations in Europe, research was conducted along a number of lines to determine the spread, genetic variation and impact that the virus was having. Serological tests were established to be able to identify rabbits that had died from RHD or had been infected, survived and built an immunological response (Capucci et al. 1996). In some of those early studies, serological testing found populations with high frequencies of RHDV-positive rabbits but no history of clinical symptoms or mortality due to RHD (Rodák et al. 1990). Subsequently a new rabbit calicivirus that was non-pathogenic was isolated in Italy and found to be genetically different to all of the known RHDV's and to differentiate it from them, it was named rabbit calicivirus (RCV) (Capucci et al. 1996). Since then, additional RCV's have been found across Europe (Chasey et al. 1995; Capucci et al. 1997; Moss et al. 2002; Marchandeau et al. 2005; Forrester et al. 2007).

Similarly in Australia during the initial monitoring of rabbit sites to assess the success of RHDV, ELISA (enzyme-linked immunosorbent assay) testing for the antibody status of rabbits revealed that cross-reacting antibodies to RHDV were found prior to RHDV arriving at some populations (Nagesha et al. 2000; Cooke et al. 2002; Robinson et al. 2002a; Bruce and Twigg 2004). The ELISA tests developed for monitoring the presence of RHDV in rabbit populations in Australia are twofold. Firstly, an antigen ELISA (cELISA) can be used to detect virus particles in tissue or sera to confirm that death of a rabbit was caused by RHD. Secondly, an antibody detecting ELISA is used to determine if rabbits have had previous exposure to RHDV and have survived forming an immunological response. The tests are carried out using methods described in Capucci etal. (1996) and classified as seronegative, seropositive (recent or previous infection, or re-infection), having maternal antibodies or benign antibodies (Table 2.1; Cooke et al. 2000). These classifications are used in determining antibody status of rabbits used in experiments presented in this thesis. Evidence of a pre-existing RHDV-related virus was also found in serology tests in New Zealand (O'Keefe et al. 1999). It was proposed that these antibodies were formed against a benign (non-pathogenic) rabbit calicivirus circulating in rabbit populations (Cooke et al. 2002; Robinson et al. 2002a) and would most likely be similar to those found in Europe (Capucci et al. 1996).

Table 2.1. Summary of immunological classifications based on cELISA, isotype titre and body weight (from Cooke et al. 2000).

	Titre*				
Class	cELISA	IgG	IgM	IgA	Notes
Negative	-	-	-	-	
Pre-existing antibodies (benign)	± (rare)	+	-	-	
Maternal antibodies	+	+	-	-	rabbits <1300g
Recent infection	++	++	++	++	IgM > 640
Past infection	+	+	±	±	
Re-infected rabbits	++	++	-	+	IgA > 160

* ++, high titre; +, low titre; -, no antibodies

This proposition proved correct and in time a non-pathogenic rabbit calicivirus (RCV-A1) was isolated from rabbits in Australia (Strive et al. 2009). This virus is more closely related to benign caliciviruses in Europe than it is to RHDV, and MRCA (most recent common ancestor) analyses suggest that the ancestor of all variants in Australia arrived in the 1850s most likely with the original imported rabbits (Jahnke et al. 2010). RCV-A1 provides incomplete, temporary protection to rabbits against RHDV (Strive et al. 2010) meaning that it may interfere in the persistence and spread of RHDV in a population. So far, RCV-A1 has only been recovered from rabbit populations from regions with a wet, cool climate in New South Wales, the Australian Capital Territory and Victoria (Jahnke et al. 2010) and may be the reason that RHDV was not as effective in these regions as it was in more arid populations.

The RCV strains isolated from across the world have also shown to provide varying level of protection against RHDV infection, ranging from full protection (Capucci et al. 1996; Capucci et al. 1997), to partial temporary protection (Strive et al. 2010) to potentially no protection at all (Marchandeau et al. 2005). Phylodynamic studies of the various strains of RCV have all placed them as diverging earlier than the appearance of RHDV, yet still in the same clade with RHDV and European Brown

Hare Syndrome when compared to other caliciviruses (Capucci et al. 1996; Forrester et al. 2007; Kerr et al. 2009; Jahnke et al. 2010). This has given rise to the theory that RHDV emerged as a virulent mutation from a pre-existing non-pathogenic RCV (Capucci et al. 1991; Cooke and Fenner 2002; Kerr et al. 2009; Kinnear and Linde 2010).

Comparisons between myxoma virus and RHDV as biocontrol agents

As seen above, myxoma virus at first caused high mortality among Australian rabbits but then began to attenuate forming many variants of lesser virulence. Rabbits also began to develop resistance to the disease and in time a dynamic process seems to have begun where field-strain myxoma viruses today kill about 50% of infected rabbits. It has only recently been accepted that the virulence of myxoma viruses is constantly changing and keeping pace with increasingly resistant rabbits in a 'biological arms race'(Fenner and Fantini 1999). However, it would be unwise to assume that such an evolutionary pattern will automatically follow in the case of RHDV. RHDV is a small RNA virus (Ohlinger et al. 1990; Valíček et al. 1990) whereas myxoma virus is a large dsDNA virus (Fenner and Ratcliffe 1965). Large complex DNA viruses are able to incorporate host genes and manipulate host defences to overcome immune and resistance mechanisms, often leading to less harm to the host (Chaston and Lidbury 2001). Small genome RNA viruses in contrast use erroneous replication to have a suite of phenotypes of which some will evade the immune or resistance response (Chaston and Lidbury 2001). This can lead to high rates of evolution as selection pressures choose new strains that are better able to

infect and cause disease in the host and this often leads to a more harmful disease as the virus has little control over the immune response (Chaston and Lidbury 2001).

The relationship between replication, transmission and virulence is also different between the two viruses and so it is likely that virulence evolution will differ as well. Myxoma virus replicates in the skin (producing lesions typical of myxomatosis) and resistance that slows disease development reduces the severity of the lesions decreasing the amount of viral replication (Best and Kerr 2000). RHDV replicates in liver hepatocytes and apoptosis or lysis to release viral particles leads to liver damage (Xu and Chen 1989; Park et al. 1991; Alonso et al. 1998). Transmission differs between the two viruses as, although both can be transmitted by arthropod vectors or direct contact, arthropod vectors are the primary route for myxoma virus (Fenner and Ratcliffe 1965) and direct contact of secretions and excretions is the primary route for RHDV (Morisse et al. 1991). Virulence in myxoma virus has evolved to an intermediate level to allow a strong enough infection to cause disease and present skin lesions, but not so severe that the rabbit dies before enough viral replication for transmission occurs and lesions appear (Fenner et al. 1956; Fenner 1983). The more positive relationship between transmission and virulence in RHDV may select for a higher virulence strain (Alizon et al. 2008).

Disease resistance and consequences for biological control agents

The use of myxoma virus to control rabbits was the first successful example of a biological control agent for a mammalian pest species. The introduction of RHDV into Australia similarly caused a successful reduction in rabbit numbers. When both of these viruses arrived in Europe, they had similar effects in reducing rabbit numbers. In contrast to the Australian situation, this caused great concern as rabbits are a native animal and an important part of the ecology of the landscape. Resistance in a host to a pathogen therefore has important implications for both the control of rabbits in countries such as Australia and New Zealand and for conservation of rabbits in Europe.

The development of resistance to a biological control agent reduces its efficacy and generally allows the host species to increase again. Rabbits began developing resistance to myxoma virus within a few years (Marshall and Fenner 1958) and by ten years after its release, the virus was killing only 50 percent of rabbits it infected in contrast to the 99 percent case mortality when first released (Fenner and Ratcliffe 1965). Rabbit populations rose and rabbits re-established in areas where they had disappeared. This again meant more money had to be spent by land managers to control rabbits using traditional, mechanical or chemical techniques. It also meant that agricultural industries could no longer support the increased stocking rates of cattle and sheep, or rely on the increased harvests seen while rabbit numbers where low. Vegetation that had been able to finally recover after being suppressed by rabbits was again heavily impacted, reducing pasture biomass and limiting the diversity of species present in a region.

The effects of increasing resistance to myxomatosis were also seen Europe. European rabbits had been suppressed by myxoma virus for nearly twenty years before resistance began developing (Ross and Sanders 1977). Farmers in Britain formed rabbit clearance societies to help keep numbers in control (McKillop 1988). Similar to the situation in Australia, myxomatosis is still responsible for the death of large numbers of rabbits (Ross et al. 1986) but rabbit numbers continued to increase and cause increasing damage (Ross and Sanders 1984).

Apart from the practical consequences of increased resistance that would be seen in a natural population (i.e. increased rabbit numbers) the understanding of the process is an important academic question providing insights into host-virus evolution, especially short term effects of evolution, and helps to test and develop evolutionary theory. Understanding the process also allows for resolution and solution of problems caused by resistance. This can either help reduce the negative impacts that rabbits have on agriculture and the environment as they do in Australia or help preserve populations to provide prey for predators or hunters as is the case in Europe.

Evolution of resistance

Experience with myxomatosis showed that under heavy selection pressure from myxoma virus rabbits rapidly developed disease resistance (Fenner and Ratcliffe 1965). Resistance in its simplest terms is a hosts ability to prevent parasite invasion and /or development (Carius et al. 2001). For resistance to establish in a population it

must have a genetic basis that can be passed on to future generations. The mechanisms of resistance can occur at any stage of viral infection to prevent or minimise the impact of disease. Therefore, it is important to understand the processes involved in viral infection. Viruses are obligatory intracellular parasites that cause strong selection for anti-viral mechanisms in the host population (Marques and Carthew 2007). The basic life-cycle of a virus can be broken into five key elements: attachment, penetration, uncoating, replication and release (Collier et al. 1998). Infection of a host occurs if the first four elements of this life-cycle are achieved (Carius et al. 2001). Exposure to a virus is merely the act of coming into the vicinity or contact with viral particles which may or may not result in infection. Resistance can prevent infection, whereby it prevents the virus reaching the stage of replication, and the host does not build an antibody response. Resistance to disease is a process whereby the host individual can initiate an immune system response quickly enough to mitigate disease symptoms as well as reducing the viral replication and release, and allowing the animal to survive.

The virus must first enter the host, usually through the mouth, nose or a break in the skin. Viruses can be passed from an infected host to a susceptible host by direct contact where behavioural traits such as grooming or food sharing can be exploited by the virus (e.g. seoul virus transmission via saliva into open wounds in rats, Hinson et al. 2004). Viruses can also be spread through inhalation of airborne particles or contact with viral particles on surfaces (e.g. influenza in humans, Bridges et al. 2003). Viruses can also exploit vectors to carry viral particles from one host to another and in some cases directly inject the virus into the host. In some cases, the virus may replicate in the vector (Mellor 1990), while in others the vector simply provides

mechanical transmission (Carn 1996). Vector transmission is the primary route of infection for myxoma virus in the European rabbit. The virus replicates in skin lesions formed by myxomatosis and these are taken up by mosquitoes or fleas. The virus is then transferred when the mosquito or flea next encounters a rabbit to feed. In the case of RHDV, transmission occurs primarily via the oral route through direct contact with secretions and excretions (Morisse et al. 1991) during grooming or feeding.

Attachment occurs by binding between the viral capsid protein and specific receptors on the host cellular surface (Collier et al. 1998). Commonly, viruses target cells in the epithelial mucosa barrier in the oropharynx and gastro-intestinal tract (Greber and Gastaldelli 2007; Nathanson 2009). Penetration occurs through either receptor-mediated endocytosis or membrane fusion (Collier et al. 1998). Non-enveloped viruses (such as RHDV) use three distinct strategies: membrane puncture, perforation or lysis (Helenius 2007) however the specific processes used by caliciviruses are poorly understood (Green 2007). Once inside the cell, uncoating (the removal of the viral capsid) releases the genomic nucleic acid in preparation for replication (Collier et al. 1998). Most DNA viruses and some negative-stranded RNA viruses replicate in the cell nucleus and need to be transported there and then imported into the nucleus (Helenius 2007). Here they utilise the cell's DNA and RNA synthesis mechanisms to provide genetic material to make copies of themselves (Collier et al. 1998). In contrast, most RNA viruses (such as RHDV) replicate in the cytoplasm and use their own RNA replicase enzymes to make copies (Collier et al. 1998). RNA replication is very fast with a single infection particle being able to, on average, produce 100 000 copies in 10 hours (Domingo and Holland 1997). RNA replication is highly error-prone due to a lack of proofreading by their replicases resulting in RNA viruses

having the highest mutation rates of any living creatures (Drake and Holland 1999). This means that an RNA virus population has many phenotypic variants that can respond quickly to selection pressures by shifting its composition, allowing for evolution to occur up to 1 million times faster than DNA-based organisms (Ball 2007). Once replication has occurred, the viruses assemble their nucleic acid and protein components to form virions that exit the cell (Pe'ery and Mathews 2007). Release from the cell can be by lysis which bursts the cell membrane and results in the death of the cell, or by budding whereby the virus is pushed out, becoming enveloped by cell membrane and resulting in the cell surviving (Collier et al. 1998).

At each of these stages of virus life cycle, there can be a number of mechanisms within the host to prevent the virus successfully achieving replication and release. Hosts that are resistant to infection will employ mechanisms that prevent attachment and penetration. Hosts that are resistant to disease will reduce replication and mitigate the signs of disease and reduce the likelihood of transmission. Animals that become infected will initiate an immune response that if successful will clear the virus allowing the animal to survive and maintain antibodies against further infection.

At the point of attachment, hosts with fewer receptors on the cellular surface will be less likely to become infected. If attachment occurs, the host's immune system begins the process of the removing the virus, and/or preventing its replication and release. The immune system can be divided into two components: the innate and adaptive immune systems, based on the properties of the cells involved and the timeframe of response (Braciale et al. 2007). The innate immune system is an immediate or very rapid response to detection of virus particles either at the cell surface or by signalling

processes from infected cells and is generally initiated by the production of interferon cytokines induced by a viral infection (Braciale et al. 2007) resulting in the destruction of virus cell particles (Biron and Sen 2007), prevention of replication (Marques and Carthew 2007), apoptosis (Everett and McFadden 1999) or activation of the adaptive immune system (Biron and Sen 2007). Intrinsic immunity is another form of innate immunity that directly restricts viral replication and assembly without inducing interferons (Yan and Chen 2012).

The adaptive immune system differs from the innate system by being specific to a viral antigen and being able to recognise and respond to a myriad of viruses (Braciale et al. 2007). A host with no previous exposure to a viral antigen will have very few lymphocytes with the specific antigen receptor required (about one in a million), but upon being activated, more of these are produced and the lymphocyte production adapts so that repeat infection is dealt with a much quicker response (Braciale et al. 2007).

These immune responses by the host drive the evolution of a viral species as they adapt to overcome them. Similarly, viruses that successfully overcome a host's defences, killing the host, mean that the strategy used by that individual was unsuccessful and may not be passed on. However, where a virus can infect, replicate and transmit out of the host quickly enough, the host will not have time to initiate an immune response and develop antibodies. In this case, immune avoidance mechanisms may be less important. Viruses can employ a number of strategies to overcome the immune system, including; interferon inhibition, suppression of RNA interference, inhibition of T-cell activity, or destruction of immune cells (Biron and

Sen 2007; Braciale et al. 2007; Marques and Carthew 2007). Myxoma virus, as an example, employs strategies of releasing a number of molecules that are homologous to natural host cellular receptors which essentially block or act as a decoy to prevent its recognition and chemicals to disrupt the cytokine networks (Upton et al. 1992; McFadden et al. 1995; Macen et al. 1996). Viruses can even exploit the immune system to their own advantage. Apoptosis is a process of programmed cell death that can be used by a host to destroy infected cells, and therefore prevent virus replication. However, if the virus can replicate before apoptosis occurs, it can use the process as a means of release and spread, and may employ mechanisms to delay apoptosis or to initiate it when replication is complete (Everett and McFadden 1999).

Recent molecular exploration for resistance to RHDV

Research in Europe, particularly France, has been attempting to establish the genes that may be responsible for resistance to RHDV. It is known that RHDV binds to the ABH histo-blood group antigens (HGBAs) found on the mucosa of the respiratory tract and duodenum (Ruvoen-Clouet et al. 2000). Similarly, human norovirus, another genus of caliciviruses that infect humans causing gastroenteritis, binds to HBGAs on the gut mucosa (Le Pendu et al. 2006). The genes that control ABH antigens and secretor status (expression or not of α1,2-linked fucose residue on the surface epithelial cells of the gut) of humans determine disease susceptibility and a single nucleotide mutation that makes the secretor fucosyl-transferase (*Fut 2*) gene non-functional is enough to provide resistance (Thorven et al. 2005; Le Pendu et al. 2006). Using this system as a model for how resistance to RHDV might be controlled in rabbits, the initial focus was on genes that control ABH antigens and secretor genes

(Guillon et al. 2009). However, those authors showed that all *Fut 2* and secretor (*Sec 1*) genes thought to be responsible for expression of HBGAs in rabbits (Lindesmith et al. 2003) are functional albeit at low levels (Guillon et al. 2009). Different strains of RHDV in France have been shown to bind to different expressed ABH antigens (Nyström et al. 2011). For example, G2 strains recognise B and H antigens while G4 strains bind to A and B antigens (Nyström et al. 2011). Those authors also demonstrated that different ABH phenotypes determined survival when exposed to low doses of RHDV, however these differences disappeared when high doses where used. This indicates that resistance may be controlled by ABH phenotype but only prevents infection by low doses of virus.

Co-evolution between virus and host

The erroneous replication seen in small RNA viruses leads to the potential for rapid evolution. Similarly, immune genes are among the fastest evolving genes (Danilova 2006). Co-evolution between a virus and its host is a process of reciprocal, adaptive genetic change as a result of selection pressure acting on one population causing selection for changes in gene frequencies in the other (Woolhouse et al. 2002). Traditionally this has been explained as an Arms Race with the 'Red Queen' hypothesis used as a metaphor whereby, as the Red Queen in Lewis Carroll's *Alice Through the Looking Glass* explains to Alice, "It takes all the running you can do, to keep in the same place", meaning that pathogens and hosts need to constantly invent new infection and protection mechanisms just to stay alive (van Valen 1973; Woolhouse and Webster 2000; Danilova 2006). Another hypothesis used to describe co-evolution between pathogens and hosts is the cost of resistance, or trade-off

hypothesis, whereby there are costs related to infection in the pathogen, and costs to the host in having immune mechanisms (Anderson and May 1982; Levin 1996; Woolhouse and Webster 2000; Alizon et al. 2008).

These theories are extremely difficult to test as selection processes occur over long time spans (Woolhouse et al. 2002) and the processes involved can be very complex. Experiments to establish resistance in rabbits against myxomatosis began from the outset of release of the virus in Australia and allowed for changes to be seen over time (Fenner et al. 1953; Myers 1954; Marshall and Fenner 1958). This allowed exploration that demonstrated that survival times of infected rabbits were increasing and virus strains were attenuating until a strain of moderate virulence become dominant in the field (Marshall and Fenner 1960; Best and Kerr 2000). Unfortunately, there were no baseline data collected from rabbit populations prior to the release of RHDV, because of the accidental manner of the introduction, and while data on mortality rates and spread were collected (Kovaliski 1998; Mutze et al. 1998; Saunders et al. 1999; Bruce et al. 2004; Story et al. 2004), there were no manipulative experiments to established to test for the development of resistance. In such cases, especially where long time-frames are involved, spatial experiments can be used to answer some of the questions arising about the co-evolutionary process because different sub-populations are at different stages of a co-evolutionary process. In these situations, spatial variation can be used to infer temporal variation (Gandon 2002; Woolhouse et al. 2002). Chapter Three of this thesis is an experiment along these lines. The European rabbit has been present in Australia for over 150 years, but has only recently been exposed to RHDV following its deliberate release in 1996. If rabbits are developing resistance against the virus then it can be expected that

different Australian populations will be at different stages of this process. This is because the initial impact of virus arrival, the timing of the arrival and frequency of epizootic outbreaks will all impact on the selection pressures applied to the co-evolutionary process. Similarly, external factors such as environmental conditions will impact on virus persistence and rabbit breeding and recruitment. Therefore, in Chapter Three, the proposition that infection rates and survival times of rabbits from different populations will differ when challenged with RHDV is tested. Support for this hypothesis will be an indication of the presence of resistance in Australian wild rabbits.

Host resistance can develop in a number of ways. At each stage of the viral infection process, from attachment and penetration of the mucosal membranes, through uptake to the replication site and replication itself, to release and transmission, the host can employ mechanisms to disrupt the process. Individuals that have a genetic or phenotypic make-up that prevents infection resulting in disease which reduces fitness or causes death will have a selective advantage over individuals that do not. The exact mechanisms involved vary from species to species and for the European rabbit, the exact processes employed are only recently being explored. Chapter Four of this thesis explores a comparison in survival curves for wild rabbits from a population with moderate resistance to RHDV against unselected domestic rabbits (i.e. rabbits with no previous history of exposure to RHDV). Differences in the survival curves will provide clues to the mechanisms of resistance and help our understanding of how resistance is developing and how this may be exploited. It is important to note that the domestic rabbits chosen for all experiments are comparable to wild rabbits. Domestic rabbits used were Crusader-type developed by CSIRO in the early 2000's by

crossbreeding New Zealand White, Californian and Flemish Giant rabbits to produce a breed (*O. cuniculus*) that maximised growth while being resistant to bacterial diseases and digestive system upset (Prayaga and Eady 2002, 2003; Eady 2008). While these rabbits grow quicker and to larger sizes than wild rabbits, they derive initially from similar stock as the wild rabbits introduced from England.

The evidence that ABH phenotype may direct survival against challenge by RHDV suggests a genetic basis for resistance. If resistance can be shown to be hereditary, this would provide further support for a genetic basis to the resistance. Artificial selection trials showed that resistance to myxoma virus was heritable (Sobey 1969). In Chapter Five an experiment is described that uses artificial selection from resistant individuals to answer the question of whether resistance against RHDV is heritable. To further explore aspects of resistance, a population was chosen that may be under stresses that make resistance in the wild a selective disadvantage. It has been demonstrated that there can be a cost to the host in having resistance (Woolhouse et al. 2002). These costs may be resource dependent (Boots 2011) and a point may be reached where the cost of having resistance is greater than the cost of the disease (Antonovics and Thrall 1994).

Of course, co-evolution means that the virus will be evolving as well through selection pressures forced on it by the resistance mechanisms of the host. The conventional view is that co-evolution between a host and pathogen will result in a stable equilibrium where the pathogen becomes harmless to the host (May and Anderson 1983). This is seen as the most advantageous situation for the pathogen as it allows more time for them to exploit their host, reproduce and transmit their progeny,

thus improving their own fitness (Regoes et al. 2000). There are many examples of this in nature, indeed, myxoma virus in its original hosts (*Sylvilagus braziliensis* and *S. californicus*) causes a persistent, benign infection with minimal cellular immune recognition and only minor disease signs (Fenner and Ratcliffe 1965; Cameron et al. 1999; Nash et al. 1999). However, there are many examples where death of the host, or at least modification of behaviour is necessary for virus transmission and it should not be expected that that pathogens evolve to be harmless to the host (May and Anderson 1983). The trade-off theory (Anderson and May 1982; Ewald 1983) states that co-evolution can follow a number of paths, driven by the relationship between virulence and infectivity of the parasite, and the cost to the host of having resistance, and these are not mutually exclusive (May and Anderson 1983; Alizon et al. 2008).

The idea that virulence and transmission are linked also led to theories of a trade-off between these two factors whereby evolution towards increased transmission has a cost in terms of duration of infection (Alizon et al. 2008). If there is a negative relationship between duration of infection and viral load, and a positive relationship between viral load and transmission, virulence will compromise towards intermediate levels (Alizon et al. 2008). This has been shown for HIV infections in humans where an increased infection time decreases the overall viral load, which reduces the transmission rate (Fraser et al. 2007). Therefore, an intermediate level of virulence, while it reduces the duration of the infection and is more harmful to the host, allows for an increased rate of transmission (Fraser et al. 2007). If there is a positive relationship between virulence and transmission, increased virulence is selected (Alizon et al. 2008). For example, *Citrobacter rodentium* infection in mice causes attaching and effacing lesions in the gastrointestinal tract resulting in diarrhoea and

transmission through the faecal-oral route (Wickham et al. 2007). Therefore, higher virulence leads to more lesions and diarrhoea and increased transmission.

The trade-off theory is an oversimplified model which has had little experimental evidence, probably owing to the difficulty in collecting data to test co-evolution and the complexities of systems (Ebert and Bull 2003; Alizon et al. 2008). Naturally selection pressures on the virus are not just related to virus activity. Host interactions also determine how infection and transmission can progress. The routes of infection and transmission can play a significant role in how virulence level evolves. In the case of the myxoma virus, transmission requires arthropod vectors to spread the virus from an infected rabbit to a susceptible one (Fenner and Ratcliffe 1965). The vector transmission is completely mechanical with no replication of virus occurring in vector (Day et al. 1956). A diseased rabbit is only infectious once skin lesions appear which contain viral particles, providing a site for vectors to take up viral particles (Fenner et al. 1956). If disease is prolonged, that is virulence is decreased, and the rabbit begins to recover, the amount of virus in the skin lesions decreases as the lesions heal, however a highly virulent strain will cause death in the rabbit before lesions can fully develop (Fenner et al. 1956). This has also contributed to the dominance of intermediate strains of myxoma virus in rabbit populations (Marshall and Fenner 1960) and explains why highly virulent strains have not persisted despite repeated releases (Berman et al. 2006).

There is often much conjecture over the term ‘virulence’, and many different definitions are used. In its broadest sense, it can be said that it is the cost to the host as a result of an infection, that is, a reduction in host fitness (Read 1994). This has little

practical application and so measurable factors such as disease-induced death rate, survival time, or sub-lethal measures such as weight loss, or reduced fecundity are used (Alizon et al. 2008). It has been put forward that virulence should be considered in terms of the pathogens genetically based strategy to exploit the hosts' resources and therefore should consider some aspect of pathogen success not just cost to host (Poulin and Combes 1999). However virulence is defined, it plays an important part in the co-evolution between a pathogen and its host.

Another factor in how virulence in a pathogen population is driven is multiple infections. Many pathogens that are normally avirulent will have virulent strains, and if multiple infections occur, it should favour increased virulence because an avirulent strain will lose the benefit of prolonged infection to exploit resources because the host will die from the infection by the more virulent strain (Frank 1996; van Baalen 1998). Other models predict that because within-cell replication and between-cell population dynamics of virus production and transmission are independent and sometimes in conflict (i.e. prolonged infection allows greater time to exploit resources for replication, but transmission may require disease or death to the host), then the virus should tend to evolve towards intermediate levels of virulence (Krakauer and Komarova 2003).

Chapter Six of this thesis is an experiment which provides the first investigation of how virulence is changing in field strains of RHDV in Australia. The implications of these outcomes are discussed in terms of co-evolutionary ideas, and the processes by which virulent forms of a virus can appear in a naive population. The methodologies

employed follow those used by Fenner and Marshall (1957) to determine virulence levels, using mortality rates and survival times of rabbits as a measure of virulence.

Conclusions

The increase in rabbit numbers seen in some populations some 15 years since the release of RHDV in Australia raised questions about the efficacy of RHDV for continued management of rabbits. To answer this concern, a number of questions were developed to better understand the system: have rabbits developed resistance against RHDV; is this resistance developing differently in different populations and what is the mechanism behind it; and is RHDV evolving in response to resistance and what direction is virulence heading. Once these are known, strategies can be put in place to overcome it to keep rabbit numbers low in Australia, or to help push resistance further to increase numbers for conservation efforts in Europe. To help frame these questions, the myxoma virus – rabbit system can be used to develop experiments and provide clues to how resistance in rabbits and virulence in RHDV may interact. It also raises questions of differences between the myxoma virus – rabbit system and the RHDV – rabbit system as they may not co-evolve in a similar manner.

In any co-evolutionary system, both the virus and host change in an attempt to keep infecting and replicating or to prevent death or loss of fitness. It is important to explore both facets of the system to understand the underlying mechanisms involved to be able to establish how the system may evolve in the future and how it may be exploited. From an aspect of rabbit control, resistance against RHDV is of highest

importance. Resistance development is to be expected as a natural consequence of host - parasite systems. The myxoma virus – rabbit system is a prime example of this and provides a model for how experiments could be designed to test for resistance against RHDV as well as allowing comparison and contrast for how resistance might be being selected for. Resistance against myxomatosis was established using direct challenge tests of wild rabbits and domestic rabbits which showed wild rabbits had a lower mortality rate, increased survival time and less severe disease symptoms (Marshall and Fenner 1958; Best and Kerr 2000). Direct challenge testing of wild rabbits with RHDV will provide information on infection rates, mortality and survival times. The use of unselected (i.e. rabbits with no history of exposure to the virus) domestic controls will provide a baseline for comparison as these rabbits would represent how the virus affected rabbits at the time of first release. Chapter Three uses direct challenge testing using the original Czech CAPM 351 RHDV released in Australia to compare resistance levels across a number of rabbit populations. Considerations had to be made for experimental procedures to take into account the nature of RHDV infection and disease. Young rabbits (< 12 weeks of age) are naturally immune to RHDV due to the presence of maternal antibodies (Robinson et al. 2002b). This meant rabbits for experiments needed to be older than 12 weeks to ensure they were fully susceptible. This natural immunity and protection of maternal antibodies in young rabbits allowed for collection of rabbits with no antibodies against RHDV from the field. By collecting young rabbits (<12 weeks of age), even if they had prior exposure to RHDV in the field, they will not have developed an immunological response to the virus and would still be susceptible to infection once older than 12 weeks of age (Robinson et al. 2002b).

As there were no challenge experiments to test for resistance in the period between the release of RHDV and the experiments presented in this thesis, comparisons can not be made to establish a temporal pattern for resistance. Spatial patterns can be used under the assumption that different populations evolve at different rates so a variance between populations can be used to infer resistance (Gandon 2002; Woolhouse et al. 2002). To exploit this model to test for resistance, rabbits were collected from a number of populations throughout central and eastern Australia to represent a range of habitats.

Chapter Four is an experiment comparing wild rabbits in Australia to unselected domestic controls against challenge with a low dose and high dose of Czech CAPM 351 RHDV. This aims at determining if a difference in infection is seen at these dose levels. If resistance is based on ABH phenotype (see Recent molecular exploration for resistance to RHDV, above) and only improves survival against low doses of virus, then we will see a difference between the domestic controls and the wild populations at the low dose but not the high dose.

In Chapter Five, the question is asked of whether resistance is heritable and can be artificially selected for. Also further explored is the possibility of a cost of resistance restricting development of resistance in a resource poor population.

In Chapter Six, the first steps are taken in determining if strains of RHDV in the field have changed in their virulence compared to the originally released strain. A

Chapter 3

Elsworth, P.G., Kovaliski, J. and Cooke, B.D. 2012. Rabbit haemorrhagic disease: Are Australian rabbits (Oryctolagus cuniculus) evolving resistance to infection with Czech CAPM 351 RHDV? *Epidemiology and Infection*, 140(11), pp. 1972-1981.

Abstract

Rabbit haemorrhagic disease is a major tool for the management of introduced, wild rabbits in Australia. However, new evidence suggests that rabbits may be developing resistance to the disease. Rabbits sourced from wild populations in central and southeastern Australia, and domestic rabbits for comparison, were experimentally challenged with a low 60 ID50 oral dose of commercially available Czech CAPM 351 virus – the original strain released in Australia. Levels of resistance to infection were generally higher than for unselected domestic rabbits and also differed (0–73% infection rates) between wild populations. Resistance was lower in populations from cooler, wetter regions and also low in arid regions with the highest resistance seen within zones of moderate rainfall. These findings suggest the external influences of non-pathogenic calicivirus in cooler, wetter areas and poor recruitment in arid populations may influence the development rate of resistance in Australia.

CHAPTER FOUR

A DOSE-INFECTION CURVE FOR RABBIT HAEMORRHAGIC DISEASE VIRUS: IMPLICATIONS FOR RESISTANCE IN WILD RABBITS.

Summary

Recent evidence of increased numbers of wild rabbits in Australia suggests the effectiveness of Rabbit Hemorrhagic Disease Virus (RHDV) as a biocontrol agent is declining. Rabbits appear to be developing resistance to RHDV, and this raises interest in both the long-term prospects for the control of rabbits in Australia and the conservation of wild RHDV depleted rabbit populations in Europe. Here, direct challenge testing is used to explore the nature of the dose-infection rate curve for RHDV in unselected domestic rabbits, and compare this with data from two Australian wild rabbit populations likely to have been selected for resistance due to repeated annual RHDV outbreaks over a decade or more. It is shown that the wild rabbits are less likely to become infected at lower doses (10^5 – 10^7 genome copies) than domestic rabbits, although all rabbits are infected and usually die when high doses of RHDV (10^8 – 10^9 genome copies) are used. This is consistent with findings from European research showing that at low to moderate doses of RHDV (10^5 genome copies) rabbit HGBA phenotype, a proposed co-receptor for RHDV infection, can determine disease outcome although this difference is not apparent at high virus doses (10^7 - 10^9 genome copies). Resistance to a low level of RHDV may allow selective advantage by allowing breeding through the duration of an outbreak and

slowing the onset of an outbreak. It is also possible that RHDV infection is more complex than a single mechanism as higher doses can overcome infection.

Introduction

The deliberate introduction of Rabbit Hemorrhagic Disease Virus (RHDV) into Australia in 1995 as a biocontrol agent against introduced European rabbits *Oryctolagus cuniculus* was highly successful (Fenner and Fantini 1999). The abundance of rabbits fell by up to 98 percent in inland areas (Mutze et al. 1998) although lesser impacts were observed in cooler coastal areas (Saunders et al. 1999; Richardson et al. 2007). RHDV causes acute liver disease characterised by necrosis of hepatocytes (Park et al. 1995) and death is associated with disseminated intravascular coagulation caused by liver break-down products (Xu and Chen 1989). The disease is usually lethal in adult rabbits (Mitro and Krauss 1993). However, rabbits that survive disease develop antibodies (Liu et al. 1984; Capucci et al. 1991; Robinson et al. 2002b) and are permanently protected from acute disease. Rabbit kittens are innately resistant to RHDV infection or disease, but from four weeks of age they become increasingly susceptible (Robinson et al. 2002b). Additionally, rabbit kittens born to females that survive the disease may also be temporarily protected against RHDV by maternal antibodies (Mikami et al. 1999; Ferreira et al. 2004) which may delay the process of kittens becoming susceptible to RHDV (Robinson et al. 2002b).

Recent evidence suggests that, apart from acquired immunity, rabbits in Australia are developing genetic resistance to infection with the Czech 351 RHDV strain originally released (Chapter Three), leading to increases in rabbit numbers in some populations (Sandell 2006; McPhee et al. 2009). Furthermore, those investigations (Chapter

Three) showed that most wild rabbits that survived challenge with a low oral dose of RHDV did not produce an antibody response and many, though not all, could be subsequently infected if again exposed to the virus. This suggests that many had been resistant to oral infection potentially reducing the rate of transmission of RHDV which is primarily via the oral route, through direct contact with secretions and excretions (Morisse et al. 1991).

Ruvoën-Clouet et al. (2000) have further shown that RHDV binds to ABH histo-blood-group antigens on the mucosa of the respiratory tract and duodenum of rabbits. This is not the main mechanism for cell-entry by RHDV, because these antigens are not found on hepatocytes where the virus primarily replicates, but it nonetheless appears to be an important step towards initial infection and may even determine the course of subsequent disease. Nyström et al. (2011) found that RHDV isolates from genetically different RHDV sub-groups (G1 - G6) bound differentially to expressed ABH antigens. For instance G4 viruses most readily bound to the mucosa of rabbits with A+B+ phenotypes rather than those expressing neither A or B. These authors also showed that differences in the phenotypic expression of histo-blood group antigens in individual rabbits determined survival when relatively low doses of virus (10^5 genome copies) were used. However, this difference disappeared at higher virus doses (10^7 – 10^9 genome copies per dose).

Nyström et al. (2011) found that in France, rabbits had been selected for a different phenotype with reduced binding capacity of G4 RHD viruses, the most common group circulating in the locality where test rabbits were obtained. They also provided evidence that Australian wild rabbits in populations heavily selected by recurrent

outbreaks of RHD show phenotypic expression of histo-blood group antigens that are consistent with the reduced mucosal binding of G2 RHD viruses. This should be expected given that the Czech 351 virus released in Australia is a G2 virus. On this basis, the evolution of resistance to RHD among Australian rabbits is expected to involve changes in ABH expression on mucosal cells of the respiratory tract and gut that reduce the capacity for RHDV particles to bind.

It is well established that human Noroviruses (NV), another calicivirus genus, also bind to histo-blood group antigens on the gut mucosa and that the genes that control the ABH antigens and secretor status of humans determine disease susceptibility (Le Pendu et al. 2006). Indeed, a single nucleotide mutation that makes the human secretor *Fut 2* gene non-functional is sufficient to provide disease resistance (Thorven et al. 2005). However, the mechanism cannot be as simple as this for RHDV resistance. Guillon et al. (2009) found that all *Fut 2* and *Sec1* alleles in rabbits were functional albeit at low levels. It is further apparent that rabbits infected via intramuscular injection, which bypasses the mucosal barrier, also show resistance to infection when low doses of virus are used (Chapter Three).

Nonetheless it is clear that the factors that reduce RHDV infection do not provide major protection. In Australia, adult wild rabbits over a year old are almost invariably sero-positive with antibodies to RHDV (McPhee et al. 2009; Mutze et al. 2010a). This means that at some stage most rabbits must encounter a sufficient dose of virus to become infected from which the animal will either succumb to RHD or survive producing antibodies against further disease.

Although studies in Australia and France have approached the issue of resistance to RHDV infection in rabbits from different directions, ideas are converging. This chapter considers how advances in understanding the genetics of rabbit resistance in France might be reflected in responses of wild rabbits in Australia to virus infectivity. This is done by considering how changes in dose – infection curves in wild rabbit populations subject to selection by RHDV appear to be diverging from the pattern shown in unselected domestic rabbit. The results were obtained by challenging both unselected domestic rabbits and wild rabbits from resistant populations with Czech CAPM 351 RHDV, the virus variant initially introduced into Australia. Evidence was found that wild rabbits are less susceptible than domestic rabbits only at low virus doses and these results are discussed and contrasted with those of Nyström et al. (2011) to broaden the conceptual model of resistance in wild rabbits.

Materials and methods

Rabbit collection and management

Domestic rabbits, more than 12 weeks old, were obtained from a commercial supplier (Mr I. Handebo, "Deeford", Armidale, NSW). All were seronegative on initial testing and because the parental 'Crusader' rabbits had previously been sourced from CSIRO (FD McMaster Laboratory Armidale NSW) they could be confirmed as having no prior exposure to RHDV (Dr. Sandra Eady, personal communication).

Wild rabbits were collected from Michelago (S35°44'43", E149°08'59") in New South Wales and Yanyanna (S31°27'17", E138°38'10") in South Australia. At both sites rabbits were trapped in wire cage-traps baited with carrot or oats. This was done

in early spring well before the expected annual outbreaks of RHDV to avoid biasing samples of rabbits towards animals with resistant phenotypes. Rabbits at both sites had been subject to repeated outbreaks of naturally occurring RHD although disease impact may have been ameliorated by the presence of a non-pathogenic lagovirus (RCV-A1) at Michelago. Prior infection with this virus gives at least temporary protection against acute RHD (Strive et al. 2009; Strive et al. 2010). Sufficient numbers of rabbits were obtained from each site for challenge tests at low virus dose (60 $I.D_{50}$) previously reported in (Chapter Three) as well as for challenge trials at a high virus dose (1500 ID_{50}).

A 1 mL blood sample was collected from each rabbit to test for previous exposure to RHDV or RCV-A1. Sera separated from the blood samples were sent to the Natural Resources Management Biosecurity Unit, Biosecurity SA, Adelaide for RHDV analysis using a series of ELISA tests: competition ELISA, and specific ELISAs to obtain IgG, IgM and IgA titres (Capucci et al. 1996), and to CSIRO, Canberra for RCV-A1 analysis (Liu et al. 2012). This enabled classification of rabbits as: seronegative to RHD, seropositive with antibodies of maternal origin, seropositive with antibodies apparently raised against RCV-A1 or seropositive survivors of RHD (Cooke et al. 2000; Liu et al. 2012).

Seropositive rabbits were euthanised while seronegative rabbits and those judged to have temporary maternal antibodies were taken to the Robert Wicks Pest Animal Research Centre, Inglewood, Queensland (Biosecurity Queensland, Department of Employment, Economic Development and Innovation) where they were held in quarantine for 10 days to ensure that none were incubating RHD, RCV-A1 or

myxomatosis. When all had reached at least 12 weeks old (body weight 900 g or more) fresh serum samples from each rabbit were assayed to confirm that they remained seronegative. Rabbits with low but equivocal traces of antibodies were considered unreliable for experimental purposes and were withdrawn from experiments. Twelve rabbits from Michelago and 11 from Yanyanna were suitable for testing at the low virus dose and similar numbers from each site (11 and 12 respectively) were tested at the high dose.

For experimental challenge, rabbits were individually housed in plastic boxes (610 x 410 x 400 mm) with insect-proof gauze lids and wire mesh floors over absorbent litter. These were held in a climate-controlled room (22ºC ± 1ºC, 50% RH, 12hr/12hr day/night cycle). Water and food (commercial rabbit pellets and fresh carrot) were provided *ad libitum*.

Virus preparation

All trials were conducted using Czech strain-351 RHDV-Batch 1B (3000LD_{50}/mL) supplied by the Elizabeth MacArthur Agricultural Institute and stored frozen at -20°C. Prior to administration, virus was thawed to room temperature and diluted in PBS to the required dose given in 0.5 mL of solution. Subsequently, real-time qRT-PCR (performed by Dr Tanja Strive at CSIRO, Canberra) was used to determine the number of genome copies in the inoculums as described previously (Strive et al. 2010). The stock Czech 351 RHDV preparation contained 3.6 X 10^8 genome copies /mL.

Determining a dose – infection rate curve for domestic rabbits

Groups of five domestic rabbits were challenged with doses of Czech CAPM351 RHDV, while holding an additional five rabbits as zero dose controls. Dose classes were achieved through dilutions of the stock Czech 351 RHDV preparation by 1:10, 1:20, 1:33, 1:50, 1:100 and 1:300. Each rabbit was administered 0.5 mL of these solutions giving dose levels (with the number of genome copies in parentheses) of 150 ID_{50} (1.8×10^7), 75 ID_{50} (9.0×10^6), 45 ID_{50} (5.5×10^6), 30 ID_{50} (3.6×10^6), 15 ID_{50} (1.8×10^6) and 5 ID_{50} (6.0×10^5). Rabbits were inoculated orally, using a 1 mL tuberculin syringe (without needle) to introduce the dose at the corner of their mouths, through the diastemma, and onto their tongues.

Experimentally inoculated rabbits were checked for signs of illness (lethargy, ataxia, death) every eight hours (i.e. at 0700, 1500 and 2300 hours) for six days then daily until 14 days post-inoculation. This 8-hour time interval allowed the best compromise between reducing stress on the animals caused by human presence while still enabling the collection of relatively fresh samples (e.g. blood for virus assay) and for the time to death to be calculated with reasonable accuracy (see Chapter Three, for method).

Data from resistant wild rabbit populations

In addition to the earlier cited results (Chapter Three) from challenging rabbits from Michelago and Yanyanna at a dose-rate of 60 ID_{50} (equivalent to 7.2×10^6 genome copies) Czech CAPM 351 RHDV (chosen from the results of the domestic dose-response curve as a dose that would infect about 66 percent of unselected rabbits, a

dose not too high that it would simply overcome resistance) we challenged rabbits from both sites with challenge doses of 1500 ID_{50} (1.8 x 10^8 genome copies) to determine infection rates. Procedures were as described for domestic rabbits.

Data analysis

Infection rate data for each group of domestic rabbits challenged were regressed against the virus dose (expressed as the equivalent intra-muscular ID_{50} dose at each dilution). Subsequently, for comparing our data with those of Nyström et al. (2011) doses were converted to virus genome copies, given that the stock Czech 351 RHDV preparation contained 3.6 X 10^8 genome copies/mL. We tested for differences between the survival patterns of Yanyanna and Michelago rabbits at high and low virus doses using the non-parametric Kaplan – Meier survival analysis (XL-STAT-Life statistics; Addinsoft SARL/Germany) and Tarone-Ware tests of equality as described in Chapter Three.

Results

Domestic Rabbits

A dose-infection rate curve was derived for unselected domestic rabbits using a series of dilutions of Czech CAPM 351 RHDV administered orally (Table 4.1, Equation 4.1). The proportion of rabbits infected (i.e. both those that died from RHD and those that survived and seroconverted) was correlated with the dose (ANOVA; F = 7.325,

d.f. = 1, p = 0.017). The regression that best fitted the data, explaining 52% of the variance was:

Infection probability (p) = 0.126 ln (dose +1) + 0.1024 (4.1)

where dose is expressed as intra-muscular ID_{50} equivalents.

Table 4.1. Infection rates of unselected domestic rabbits (n = 5 for each dose level) orally challenged with different doses of RHDV. All infected animals died from RHD.

Dose $I.D._{50}$	Total proportion infected
0	0
5	0.20
15	0.60
30	0.40
45	1.00
60*	0.67
75	0.80
150	0.80

* From Chapter Three

The fitted curve for domestic rabbits rises very sharply with low increments in viral dose but then flattens out with the last few rabbits requiring relatively high doses of RHDV for infection (Figure 4.1).

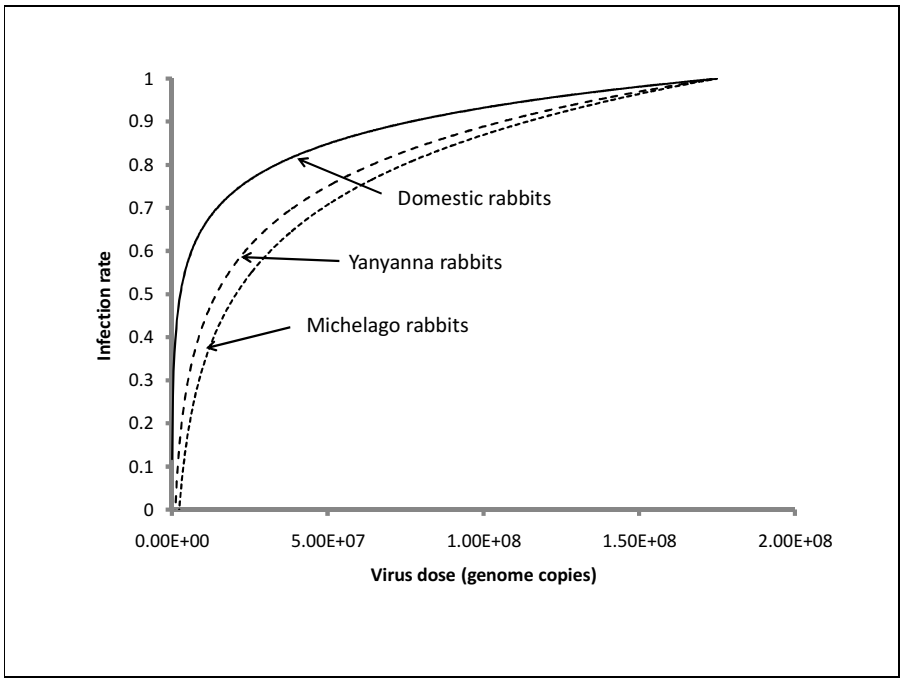

Figure 4.1. Schematic representation of the dose-infection rate curves for unselected domestic rabbits and the likely dose-infection rate curves for wild rabbits from Michelago and Yanyanna.

Significance of differences in responses between wild rabbits dosed at 60 and 1500 ID_{50}

Although only two groups of rabbits from Michelago and Yanyanna were compared using different virus doses, there were clear differences in responses as shown in Figure 4.2 (Tarone-Ware test of equality, Michelago $p < 0.001$, Yanyanna $p = 0.0001$).

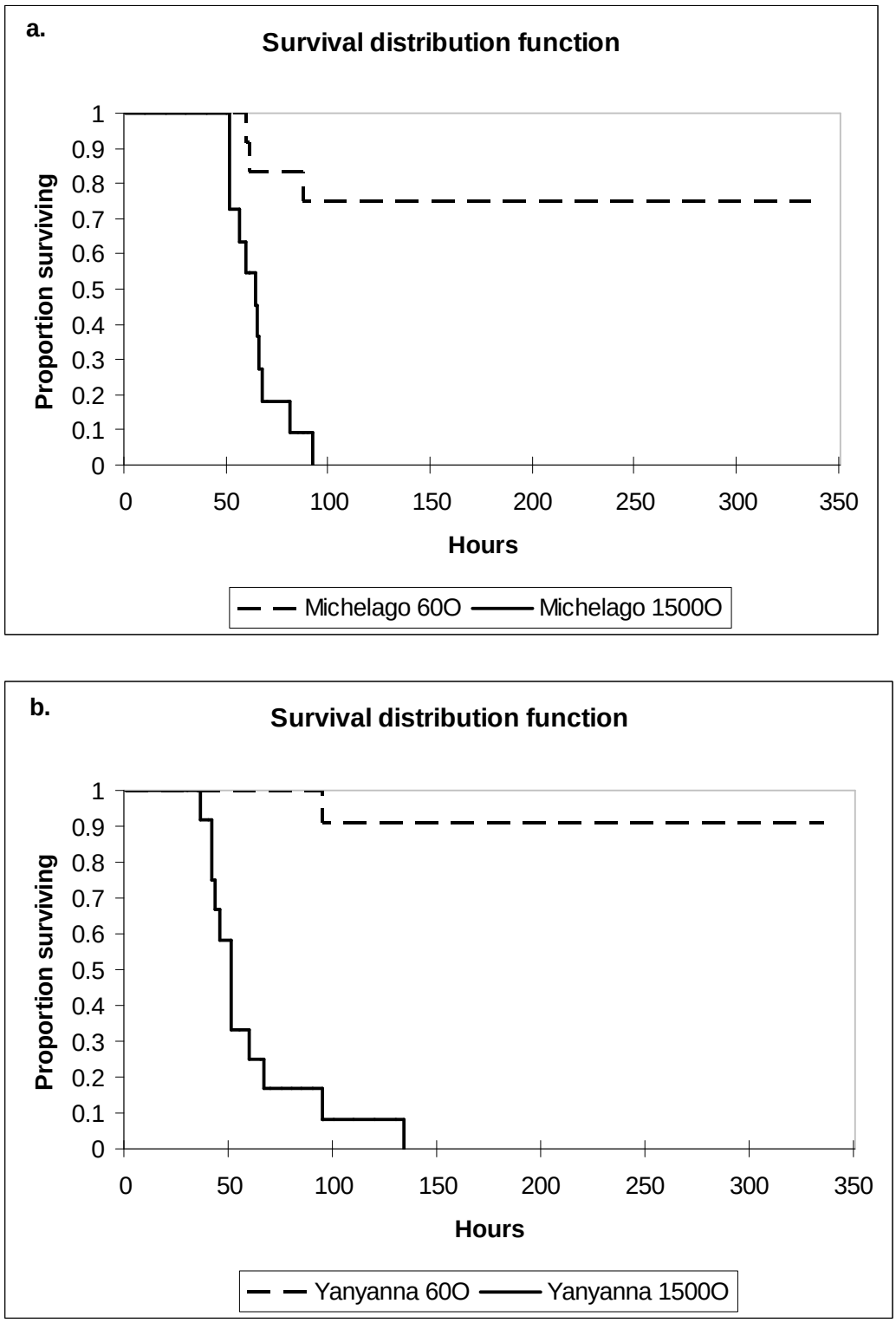

Figure 4.2. Differences in survival of **a.** Michelago rabbits and **b.** Yanyanna rabbits dosed orally with 60 ID_{50} and 1500 ID_{50}. At the low virus dose, 8 of 11 Michelago and 10 of 11 Yanyanna rabbits survived to the end of the two week trial period (336 hr) but all those given a high dose died within 6 days (144 hr).

Comparisons of infection rates between domestic and wild rabbits

Results for wild rabbits from Michelago and Yanyanna showed that responses differed markedly from those of domestic rabbits at low virus dose (60 ID_{50}). The proportion of rabbits infected at Michelago, 0.25, and Yanyanna, 0.36, was significantly lower than the infection rate of 0.67 observed among domestic rabbits (Chapter Three). By contrast, as is the case for domestic rabbits (Lenghaus et al. 1994), all wild-caught rabbits, 11 from Michelago and 12 from Yanyanna, became infected and died at the high virus dose of 1500 ID_{50}. For the wild rabbits, dose response had apparently been altered at low virus dose levels despite no evidence of increased resistance to infection at very high virus doses. The results are shown in Table 4.2 and schematically in Figure 4.1. The schematic survival curves for the wild populations were based on a semi-log equation as for domestics, adjusted for the lower infection rate at the low dose.

Table 4.2. Comparison of infection rates of rabbits from different sources at two doses of Czech RHDV. Note that only one out of the four Yanyanna rabbits at the 60ID_{50} dose rate that became infected died, but all of the Michelago and domestic rabbits that became infected died and all the wild rabbits that became infected at the 1500ID_{50} dose rate died.

Origin of rabbits	Dose rate	
	60 ID_{50}	1500 ID_{50}
Domestic	0.67* (n = 12)	1.00†
Michelago	0.25* (n = 12)	1.00 (n = 11)
Yanyanna	0.36* (n = 11)	1.00 (n =12)

*From Chapter Three.
† Well established from previous laboratory experiments and other references e.g. Lenghaus *et al*. (1994).

Discussion

The shapes of dose-infection curves depend on many factors involved in the interaction between the pathogen and its host. Often, infection relies on a threshold level of pathogen being reached. This results in a low infection rate at low doses, then a rapid increase in infection at the threshold level up to the maximum infection level, producing a sigmoidal dose-infection curve (Regoes et al. 2003). Foot and Mouth Disease virus infection of cattle and sheep, for example, follows this sigmoidal pattern (French et al. 2002).

The dose-infection curve for RHDV in unselected domestic rabbits is unusual because the probability of infection rises very quickly at very low doses of virus but large doses are required to infect all rabbits. For example, we know from our experiments that the oral dose infecting 50% of domestic rabbits (2.6×10^6 genome copies) needs to be more than doubled to infect 60% of rabbits and it takes very high doses, about 1500 ID_{50} (1.8×10^8 genome copies) to consistently infect all rabbits. Indeed, the virus dose in the commercially available virus preparation from the Elizabeth Macarthur Agricultural Institute was established on that basis.

During the early stages of RHDV transmission in Australia following the release, the unselected wild rabbits would have been susceptible to low levels of viral particles being transmitted. Early trials showed that even two to six LD_{50} units of RHDV on one to two flyspots were sufficient to infect and kill wild rabbits (Asgari et al. 1998). Obviously the virus is highly contagious which may have been responsible for the

dramatic declines seen in populations when the virus first arrived (Bowen and Read 1998; Mutze et al. 1998) and the speed at which it spread (Kovaliski 1998). However, the need for a high dose of virus to ensure infection of all animals will mean that some will survive outbreaks, become immune and continue breeding. This is especially so if those animals are exposed to a lower dose during the early part of an epizootic event.

Importantly, the doses we used to challenge wild rabbits (60 ID_{50} or approximately 7.2 x 10^6 genome copies) are within the mid-range of the doses Nyström et al. (2011) used in experimental challenge. It is important to note however that viral copy numbers do not necessarily equate to the amount of infectious particles present in a virus preparation as the ratio of infectious particles to viral copies can vary between virus preparations. The doses used are also at about the point on the dose-infection rate curve for unselected domestic rabbits where the curve begins to flatten out noticeably. Below that level, i.e. when 10^5 genome copies were used, infection rates fell sharply with declining dose.

At similar doses, Nyström et al. (2011) showed that the outcome of infection was modified depending on rabbit HBGA-phenotype. However, rabbit HBGA-phenotype did not influence disease outcome when higher virus doses of 10^7 and 10^9 genome copies were administered.

Together with the data provided by Nyström et al. (2011), our observations of reduced infection rates in Australian wild rabbits are best explained in terms of reduced capacity of RHDV to bind to cells of the rabbits' mucosal surfaces. This is further supported by the fact that the most common AB phenotypes in Australian rabbits are

those that might be expected if there had been selection of rabbits which resisted ready binding of a G2 RHDV such as the Czech strain virus originally released in Australia (Nyström et al. 2011).

Despite the strong evidence that rabbits in Australia are developing resistance to infection, it is evident that most rabbits still become infected with RHDV during the first year of their lives; in most rabbit populations over 95% of breeding adult rabbits are seropositive (McPhee et al. 2009). There are two potential explanations for this. The simplest idea is that rabbits eventually encounter a very large dose of virus (e.g. by direct contact with a cadaver in a warren, or an increasing dose during an outbreak which may lead to survival of infection, and seroconversion, rather than avoidance by resistance). Secondly, it is possible that circulating viruses have sufficient diversity for some variants to bind despite selection of resistant rabbit phenotypes. This second possibility seems more likely in Europe, where several distinguishable sub-groups of RHDV (G1 – 6) circulate (Le Gall-Recule 2003), and these are known to differ in their capacity to bind to expressed HBGAs (Nyström et al. 2011). However, it seems a less probable explanation in Australia where virus variants are still clearly recognisable as derivatives of the G2 Czech strain RHDV originally introduced.

In this sense, it seems that resistance to infection in wild rabbits is more likely to delay rather than prevent infection. One possible advantage of delaying infection may be that it would allow survival during the early periods of virus outbreak when low levels of viral particles are circulating. This would allow reproduction to continue and allow those offspring a better opportunity to survive the outbreak. This is especially so because rabbits infected when young have a much higher chance of surviving

infection than rabbits older than 12 weeks (Robinson, et al. 2002b). It may also slow the onset of an outbreak as there are fewer rabbits susceptible to lower doses of virus and viral replication will be restricted until such time as sufficient viral particles are present to include the resistant rabbits in the susceptible population.

If resistance selection is leading to countering virus particles at low doses binding to ABH antigens, there must be an advantage in slowing the uptake of viral particles. To explore this idea further it would be useful to have more information on the infection process that follows the initial binding of viral particles onto ABH antigens on the mucosal surface. At this stage we only know that after infection of unselected rabbits viral plus- and minus-strand RNAs are soon detected within the cytoplasm of hepatocytes and are also found in apparently morphologically intact Kupffer cells and splenic and alveolar macrophages (Ramiro-Ibanez et al. 1999; Kimura et al. 2001). We also know that these processes take place very quickly. Guittré et al. (1996) found that viral RNA was detected in the liver and spleen by RT-PCR within 18 hours in rabbits intra-nasally inoculated with RHDV. Marques et al. (2010) have also shown that there is acute depletion of lymphocytes, mainly B and T cells, associated with apoptosis during the early phase of RHDV infection and this occurs well before the gut and pulmonary lymphoid tissues generally become involved; the tonsils and lymph nodes are only RT-PCR positive after 36 hours.

If, as we suggest, there are alternative pathways for infection, these would presumably be more advantageous for the rabbit. We do not understand what such alternative pathways might be but there are some interesting possibilities. For example, Gebert and Posselt (1997) showed that M-cells of the caecal lymphoid patch express high

levels of α-1-2-fucose (potentially important for binding RHDV) and these cells also take up peptides, viruses and bacteria from the gut lumen by cytosis, passing these into the lymphoid dome where they are available to antibody presenting cells. Lymphoid tissues in the hind gut could therefore provide an alternate pathway of infection.

Controlled lymphocyte dissemination of RHDV might be less risky than rapid virus-mediated infection across the oral and pulmonary mucosa if virus spread is slowed and antibody responses are elicited as early as possible. Indeed, there is some evidence for this because wild rabbits from populations showing resistance to infection with RHDV have extended survival times, up to 135 hours compared with an average of 60 – 70 hours in unselected rabbits, if they become acutely infected (Chapter Three). This supports the idea that a secondary infection pathway may allow rabbits more time to mount an appropriate immune response. The case-fatality rate nonetheless remains high in experimentally infected rabbits (87%; Chapter Three) suggesting that, at this stage of the evolution of resistance, the selective advantage in channelling infection through a secondary pathway must still be small.

An important role of lymphocytes in controlling disease development could also explain age-specific disease outcomes in RHDV-infected rabbits, especially the observation that young rabbits a few weeks old are far less susceptible to acute RHDV infection than older rabbits (Mikami et al. 1999; Ferreira et al. 2004). Tomai et al. (1992) found that the macrophage and lymphocyte functions of young rabbits (2 – 4 weeks old) are intact but down-regulated making them less likely to take up foreign proteins or pathogens.

The data presented here generally complement the observations of Nyström et al. (2011) reinforcing the idea there has been genetic selection for resistance to RHDV in wild rabbits. However, a closer understanding of infection pathways and especially any differences in the infection processes between naive and resistant rabbits would be extremely useful in establishing why reduced binding of RHDV to ABH antigens appears to provide a selective advantage.

From the perspective of the virus, it seems there must be a selective advantage in being able to mediate attachment and entry through the most advantageous route and avoid those routes where infection is better controlled by the host and less likely to produce acute infection and further transmission. It may also be that if resistance mechanism in rabbits is geared towards avoiding infection by reducing binding capacity, they may effectively be reducing the infectious dose they encounter. In this case, larger amounts of circulating virus may be required to cause sufficient uptake.

CHAPTER FIVE

RESISTANCE SELECTION AGAINST RABBIT HAEMORRHAGIC DISEASE VIRUS IN EUROPEAN RABBITS (*Oryctolagus cuniculus*)

Summary

Experimental evidence indicates that European rabbits in Australia are developing genetic resistance to infection with rabbit haemorrhagic disease virus. To further support this hypothesis, an experiment to demonstrate that such resistance is heritable and can be artificially selected is described. For this work, wild rabbits from a population that showed relatively low resistance to infection (Bulloo Downs, Queensland) were challenged with a low dose of RHDV ($60ID_{50}$) and survivors paired to breed subsequent generations which in turn were again challenged with RHDV. Despite experimental problems including lower than expected survivorship among the rabbits challenged, rabbits resistant to infection with RHDV were quickly selected, confirming heritability of resistance. These results pose new questions, the most obvious one being that, if rabbits have the genetic variability to enable rapid selection for resistance to RHDV infection, why was the Bulloo Downs population demonstrably no more resistant to infection than unselected laboratory rabbits? Possible explanations for this, such as the cost of selection in arid, resource-poor environments are considered along with the implications of this work for future biological control of rabbits in Australia.

Introduction

Wild European rabbits (*Oryctolagus cuniculus*) are a serious agricultural and environmental pest in Australia. The introduction of rabbit haemorrhagic disease virus (RHDV) in 1995 greatly reduced their numbers in many populations (Bowen and Read 1998; Mutze et al. 1998) bringing strong economic and biodiversity benefits. However, recent evidence of increased rabbit numbers (Sandell 2006; McPhee et al. 2009) and the demonstration of resistance in some populations (Chapter Three) raises questions about the long-term efficacy of RHDV as a control tool for rabbits.

Resistance develops as a consequence of selective pressures from a pathogen (Woolhouse et al. 2002). Individuals with a genetic advantage against the pathogen will have better reproductive success than individuals that don't and over time the proportion of resistant individuals in a population will increase. The rate at which resistance develops depends on a number of factors including the selection pressure by the pathogen and interactions between pathogen and host.

Rabbits under selection pressure from myxoma virus developed noticeable disease resistance within a few years of its release in Australia (Marshall and Fenner 1958). In wild rabbits myxomatosis now develops more slowly than in unselected domestic rabbits (Best and Kerr 2000). Survival time has not only increased, but disease severity is decreased as well (Best and Kerr 2000). This indicates that resistance slows the development of disease and allows the rabbit to better control the infection. At this stage, however, resistance to RHDV appears to prevent infection at low virus dose, potentially delaying infection, rather than markedly reducing the severity of disease (Chapter Four).

It is known that RHDV binds to histo-blood group antigens on the mucosal lining (Ruvoen-Clouet et al. 2000), and recent research has shown that ABH antigen phenotype determines survival against low dose challenge of RHDV (Nyström et al. 2011). Selection for phenotypes that resist binding by RHDV could increase the proportion of resistant individuals in a population.

Selection for resistance may not just involve selection pressures by the virus. It has been demonstrated that there can be costs to the host in having resistance to a pathogen (Woolhouse et al. 2002). At some level, the cost becomes greater to the host than the benefit of not being affected by the virus, especially in the absence of disease, and resistance will not evolve beyond that point (Antonovics and Thrall 1994). These costs can be a direct detrimental effect on the host due to the biochemical, morphological or phenological features that confer the resistance (Antonovics and Thrall 1994) and may lead to reduced survivorship, reduced fertility, reduced competitive ability or increased susceptibility to other pathogens (Woolhouse et al. 2002). An example of the cost of resistance is seen in resistance to the parasite *Schistosoma mansoni* by the snail *Biomphalaria glabrata*. In this host-parasite system, snails selected for resistance against *S. mansoni* had lower reproductive success in that they produced fewer surviving offspring than non-resistant snails (Webster and Woolhouse 1999). This difference was seen whether the parasite was present or not, so while being able to survive infection and reproduce when the parasite is present, it is a selective disadvantage when the parasite is absent and non-resistant snails will produce more offspring and contribute more genetic material to the population (Webster and Woolhouse 1999).

These costs may be resource dependent and when conditions are good the costs to the host may be of little consequence, however when conditions become marginal, the costs may be detrimental to the hosts survival or reproduction (Boots 2011). For rabbits living in environments that are not ideal, or are under stress, having resistance to RHDV may provide a cost that becomes greater than the protection afforded by it.

Here, the possibility for artificial selection for resistance to infection in rabbits under laboratory conditions is explored. This would show that the resistance is heritable. In experiments to assess the heritability of resistance to myxomatosis, Sobey (1969) selectively bred from rabbits that had survived challenge with myxoma virus and a similar approach will be used here. By using a population of rabbits with a low resistance level from a site under environmental and external stress inferences of a cost to resistance can be explored.

Materials and methods

Experimental design

For this selection experiment, rabbits were obtained in 2009 from Bulloo Downs, Queensland. Rabbits from this site were as susceptible to RHDV infection as unselected domestic rabbits (Chapter Three). From eight of the rabbits caught, a generation of young rabbits was bred to provide an initial generation for challenge. As for previous trials, all were reared to at least 13 weeks of age when maternal antibodies have been lost and rabbits have lost natural juvenile resilience (Robinson et

al 2002). These two groups (named generation 0 for the original wild caught rabbits and generation 1 for the laboratory bred rabbits) were simultaneously challenged with a dose of $60ID_{50}$ RHDV. Comparisons were made of infection rates, mortality and survival times.

Initially it was planned that the rabbits that survived this first challenge would be used to breed subsequent generations whereby generation 0 survivors would breed a generation 1b group, and generation 1 survivors would breed a generation 2 group. This would have allowed comparison between generations 1 and 2 and generations 1b and 2 to show if resistance was increasing as a result of breeding only from survivors of RHDV challenge. Unfortunately, the mortality rates in the first challenge were higher than expected from previous experiments (Chapter Three) for the generation 0 and generation 1 groups and insufficient numbers of each sex survived to allow breeding of each group. Therefore the decision was made to mix the survivors of the two generations to breed the generation 2 group. The generation 2 rabbits were reared under laboratory conditions and again challenged when at least 13 weeks of age.

Unselected domestic rabbits provided a comparison to the wild rabbits. The breeding programs and challenge tests were to be the same as for the wild rabbits, however, although test generations 0 and 1 were produced, the same problem was encountered with higher than expected mortalities. This resulted in only one survivor which precluded any further breeding. To compensate, five additional domestic rabbits were obtained for use as experimental controls when the generation 2 wild rabbits were tested.

Prior to both challenge tests, a blood sample (~1 mL) was taken from each rabbit and serum separated for analysis by ELISA (performed by Biosecurity SA) to establish their antibody status against RHDV. Upon death or 14 days post challenge for survivors, a blood sample was again taken for ELISA analysis, this time to confirm cause of death (virus capture ELISA) or seroconversion (competition ELISA).

Source and care of rabbits

Wild rabbits (n = 20, 10 male, 10 female) were live trapped from Bulloo Downs station, south-western Queensland and transported to the Robert Wicks Pest Animal Research Centre, Inglewood, Queensland. All rabbits were ELISA tested (antigen detection ELISA) to ensure they had no antibodies to RHDV. These wild-caught rabbits represent generation 0 for the purposes of experiments. Eight of these rabbits were randomly chosen and separated into breeding pairs and housed in a temperature controlled laboratory (20 ± 1°C, day/night cycle 12hr / 12hr) in large cages. Twenty young (10 males and 10 females) born to these rabbits were reared until 13 weeks of age, representing generation 1 for the experiments. All rabbits were fed commercial pellets and water *ad libitum*, and carrots twice weekly. For experimental challenge, rabbits were individually housed in plastic boxes (610 x 410 x 400 mm) with insect-proof gauze lids and wire mesh floors over absorbent litter. These were held in a climate-controlled room (22ºC ± 1ºC, 50% RH, 12hr / 12hr day/night cycle, lights on: 06:00). Water and food (commercial rabbit pellets and fresh carrot) were provided *ad libitum*. Survivors from the first RHDV challenge were bred to produce a second generation (generation 2).

Twenty domestic rabbits (10 male, 10 female), more than 12 weeks old, obtained from a commercial supplier (Mr I. Handebo, "Deeford", Armidale, NSW) were used as experimental controls when wild rabbits were challenged. All were seronegative on initial testing and because the parental 'Crusader' rabbits had previously been sourced from CSIRO (FD McMaster Laboratory Armidale NSW) they could be confirmed as having had no prior exposure to RHDV (Dr. Sandra Eady, CSIRO, personal communication). These rabbits were used as generation 0, and eight of these were used to breed generation 1 as described for the wild rabbits. An additional five rabbits were subsequently sourced from the same supplier to provide controls when testing generation 2 of the wild rabbits.

Virus preparation and administration

For challenge tests with generation 0 and generation 1 rabbits, a 1:25 dilution of commercially available RHDV in PBS was prepared. The virus, (Czech CAPM 351 RHDV, batch 1-C, from Elizabeth Macarthur Agricultural Institute, Camden, NSW) was a different batch number, 1-C, and so may have differed slightly from batch 1-B used in previous experiments (Chapter Three and Chapter Four). Nonetheless, the basic undiluted stock solution was standardized to contain 3000 rabbit infectious doses mL^{-1} (as for batch 1-B) so the dose used in our trials was equivalent to a nominal intramuscular dose of $60ID_{50}$ when delivered in a volume of 0.5 mL (see Chapter Three and Chapter Four for rationale). Each rabbit received 0.5 mL of this virus solution administered orally by a tuberculin syringe (without needle) placed through the diastema onto the back of the tongue.

Monitoring and sampling

Following inoculation, rabbits were checked at eight hour intervals (nominally 0700, 1500 and 2300 hours) to minimise disturbance while still allowing time of death to be calculated and blood samples to be collected. Upon death, rectal temperature and a blood and liver sample were taken. Rectal temperature was used to calculate time of death since the previous check time (see Chapter Three for method). Blood samples were tested (performed by J. Kovaliski, Biosecurity SA) using antigen (virus capture) ELISA to confirm RHDV and liver samples held for future tests if required. Rabbits surviving to 14 days post-administration were blood-tested to assess antibody status and were randomly paired to breed generation 2. The second challenge, conducted when all rabbits in generation 2 were at least 13 weeks old, involved the survivors of the first challenge generation 2 as well as five unselected domestic rabbits as controls. Procedures were the same as for above.

Results

From the first challenge, 90% of rabbits from generation 0 and 80% of rabbits from generation 1 died from rabbit hemorrhagic disease (RHD) as shown from ELISA testing (Table 5.1). All eight of the rabbits used to breed generation 1 died. All of the generation 0 domestic controls and 95% of the generation 1 domestics died from RHD (Table 5.1). None of the surviving rabbits showed an antibody response to RHDV in ELISA testing. There was no significant difference between the mortality rates (X^2 = 5.479, d.f. = 3, p = 0.140) or survival times of any of the generations for wild and domestic rabbits (ANOVA, F = 0.742, d.f. = 4, p = 0.566).

Due to the low numbers of survivors and no males surviving from generation 0, the remaining six wild rabbits (2 females from generation 0, 2 females from generation 1 and 2 males from generation 1) were paired so that the two males were mated with two females (one from generation 0 and one from generation 1) each. One of these pairs (a female from generation 1) did not produce any offspring so all of generation 2 came from the other three pairings. Unfortunately with only one domestic rabbit, this line could not be continued.

In the second challenge, the survivors of the first challenge (generation 0, n = 2; generation 1, n = 4) all survived again, and all twenty of generation 2 survived as well (Table 5.1). None of the survivors of the second challenge, including those that previously survived the first challenge, showed an antibody response to RHDV in ELISA testing. Four out of five of the control rabbits died (average survival time 61.2 hours). This did not differ from expectations and confirmed that the virus preparation was infective for unselected rabbits.

Table 5.1. Rabbit mortality and survival times for each generation of rabbits challenged with RHDV.

	Wild rabbits				Domestic rabbits		
	Challenge 1		Challenge 2		Challenge 1		Challenge 2
	generation 0	generation 1	generation 0 and 1 survivors	generation 2	generation 0	generation 1	challenge 2 controls
Number challenged	20	20	6	20	20	20	5
Number died	18	16	0	0	20	19	4
Average survival time (hours) ± SE	57.2 ± 5.94	58.7 ± 6.00	n.a.	n.a.	57.5 ± 5.92	56.8 ± 5.47	61.2 ± 6.23

Discussion

In a tightly controlled laboratory situation, it appears that selection for resistance to infection with RHDV can be rapid. Breeding from five individuals that had shown resistance to infection with a low dose (60 ID_{50} RHDV produced a generation that all withstood challenge with a low dose of RHDV and which did not elicit an antibody response.

Nonetheless, the use of only five individuals for breeding the second generation was not ideal and may confound the result. It is possible that the result is an outcome of genetic drift, as so few rabbits were available to breed, rather than actual selection. From earlier experiments (Chapter Three) more survivors were expected from the first challenge and a larger initial number of rabbits may have provided more survivors to allow a breeding group with greater genetic diversity. Nonetheless, the results strongly suggest that some rabbits even in a highly susceptible population can resist challenge with a low dose of 60 ID_{50} RHDV and that this resistance is heritable.

The mortality rates of generations 0 and 1 for the wild rabbits was similar to that of the domestic rabbits and confirmed the results previously obtained (Chapter Three) that the Bulloo Downs population is no more resistant to challenge with a 60ID_{50} RHDV dose than unselected domestics. That the wild population of rabbits at Bulloo Downs shows very little resistance when it is clear from the results here that

resistance could develop quickly raises the question of why it hasn't. There is a curvilinear relationship between resistance and rainfall (Chapter Three) whereby resistance is lower in arid regions and regions with high rainfall. While the presence of the non-pathogenic rabbit calicivirus (RCV-1A) at the wetter sites (Jahnke et al. 2010; Strive et al. 2010) may explain why resistance develops more slowly, at the arid sites it may be a lack of resources that interferes with resistance development by reducing recruitment (Chapter Three).

Another possibility for resistance not being selected for in arid sites is that there may be a cost of resistance (Woolhouse et al. 2002) to the rabbit in having resistance which is not favoured when conditions are poor. Costs of resistance can be a direct detrimental effect on the host resulting from biochemical, morphological or phenological features that confer the resistance (Antonovics and Thrall 1994) and may lead to reduced survivorship, reduced fertility, reduced competitive ability or increased susceptibility to other pathogens (Woolhouse et al. 2002). These costs may be resource dependent and when conditions are good the costs to the host may be of little consequence, however when conditions become marginal, the costs may be detrimental to the hosts survival or reproduction (Boots 2011). Such costs to rabbit resistance to RHDV may be of little consequence in populations from regions with moderate to high rainfall but may impact on populations from arid regions.

This rapid change in susceptibility to infection differs from the situation seen in domestic rabbits selectively bred for myxoma virus resistance where even after three generations, the best recovery rates for KM 13/2 strain was 80% (Sobey 1969). Resistance breeding against myxoma virus was complicated by environmental factors

as well as consequences of disease that prolonged the time between infection and when a survivor could be used for breeding owing to reduced fertility (Sobey 1969). Survival from challenge with RHDV appears not to be as complex as survival from myxomatosis, as challenges using low RHDV doses (Chapter Three) resulted in many rabbits simply not becoming infected and thus there are no signs of disease or reductions in fertility.

The indications that resistance is heritable lends weight to the findings that ABH phenotype determines survival against low doses of RHDV (Nyström et al. 2011). Rabbits with the appropriate ABH-type for the endemic strain of RHDV may have a selective advantage and be able to pass on that trait to offspring increasing the overall resistance of a population.

The continued usefulness of RHDV as a biocontrol agent in populations may be limited if resistance to infection is developing as the proportion of resistant individuals may be able to increase quickly as the data presented here suggests. In these populations additional control techniques such as warren ripping, poisoning or fumigation may need to be employed to limit rabbit recovery. Rapid increase in resistance levels does not appear to be occurring in the wild, however, which may be the result of a cost of resistance. In regions where rabbits are affected by resource restrictions which may increase the cost of resistance, releases of RHDV to keep virus activity in the population may keep these populations repressed.

CHAPTER SIX

VIRULENCE OF FIELD ISOLATES OF RABBIT HAEMORRHAGIC DISEASE VIRUS IN RESISTANT WILD RABBITS: A PRELIMINARY INVESTIGATION

Summary

The highly pathogenic rabbit haemorrhagic disease virus is a relatively new virus in the European rabbit and even more so in Australia where it was deliberately introduced in 1995 as a biocontrol agent. This virus was highly virulent at the time of introduction, causing high mortality rates in most populations. Recent evidence of increases in rabbit numbers and resistance in some populations raises questions as to whether rabbit resistance is limiting the effectiveness of RHDV as a biological control agent and whether the virus in turn is changing to maintain its capacity to persist in these populations. No research has been conducted on how the virulence of RHDV may have changed since its release in Australia. Here a comparison is made between field strains of RHDV collected from 2006, 2007 and 2009 and the commercially available Czech 351 strain originally released in Australia. This demonstrates for the first time that there is selective pressure for the virus to maintain high levels of virulence, to overcome developing genetic resistance Australian wild rabbits. The findings highlight the importance of mechanical insect transmission of RHDV and the dead rabbit as the main source of virus transmission. This is an important contribution to understanding host-pathogen co-evolution of rabbits and RHDV.

Introduction

Rabbit haemorrhagic disease virus (RHDV) was released in Australia in 1995 and generally caused large reductions in rabbit numbers (Mutze et al. 1998, Bowen and Read 1998). Recent evidence of increasing numbers of rabbits (Sandell 2006; McPhee et al. 2009) and the development of resistance to RHDV infection in some populations (Chapter Three) raises the question of whether RHDV will loose its capacity to infect rabbits or if it is changing to maintain a high or intermediate level of virulence.

The complexity of virulence evolution has given rise to a number of models of how virulence evolution should be directed. So called 'conventional theories' or avirulent theories of virulence evolution (May and Anderson 1983), whereby parasites evolve to become harmless to their host are being replaced by models that incorporate more of the fundamental interactions between a parasite and its host (Alizon et al. 2008). The trade-off theory includes the interactions of three epidemiological parameters: the virulence of the parasite, the transmission rate of the parasite and the recovery rate of the host (Anderson and May 1982; Ewald 1983). One of the fundamental problems in discussions of these issues is the definition of virulence (see Chapter Two). Ultimately, virulence is a product of the parasite-host interaction, treated as a product of the parasite, but measured through the effect on the host (Poulin and Combes 1999). In the broadest sense, it is the cost to the host due to infection (Read 1994).

According to the trade-off theory, transmission and virulence are positively coupled until they negatively affect host recovery, suggesting that some degree of virulence is an inevitable component of most host-pathogen interactions (Levin 1996; Alizon et al. 2008). Other theories, such as the 'short-sighted' evolution theory suggest virulence in some cases constitutes a by-product, where mutations that increase virulence convey a competitive advantage in a pathogen population, but not beyond the current host (Levin and Bull 1994). The high levels of virulence shown by certain soil bacteria such as *Bacillus anthracis* or *Clostridium tetanii* that occasionally affect vertebrate hosts is explained by the co-incidental hypothesis of virulence evolution, as the accidental disease caused by these agents that are not mandatory parasites but free living organisms is neither required nor beneficial for their survival (reviewed in Levin 1996).

One of the most well studied co-evolutionary systems is the myxoma virus in the European rabbit (*Oryctolagus cuniculus*) in Australia. Myxoma virus was introduced into Australia in 1950 as a biocontrol agent for the management of rabbits (Fenner and Ratcliffe 1965). It had a devastating effect on rabbit populations, causing reductions of up to 99% (Fenner et al. 1953). Within a few years, highly attenuated strains of myxoma virus were seen, although these were never dominant and quickly disappeared (Fenner and Marshall 1957; Edmonds et al. 1975; Fenner 1983). In the field, virus strains with moderate virulence persisted and became dominant, even remaining after the introduction of the highly virulent Lausanne strains (Fenner 1983; Berman et al. 2006) . Fenner (1983) described the reason for this as being caused by the virus' need to cause disease in the rabbit to produce skin lesions so viral particles could be taken up and transmitted by arthropod vectors, while not being so virulent

that death of the rabbit occurred too soon for enough lesions to produced. Notably, similar adaptative processes were observed on the European continent following the introduction of myxoma virus (Fenner and Chapple 1965; Fenner and Ratcliffe 1965; Kerr et al. 2012). The predominance of myxoma virus strains with intermediate levels of virulence has been used as evidence for the trade-off theory of virulence evolution (May and Anderson 1983; Levin 1996).

The evolution of virulence in RHDV may be quite different to that seen for myxoma virus as RHDV is very different virus to myxoma virus. RHDV is a small positive-stranded RNA virus (Ohlinger et al. 1990) whereas myxoma virus is a large DNA poxvirus (Fenner and Ratcliffe 1965). Myxoma virus emerged in the European rabbit having crossed from other lagomorphs (Fenner and Ratcliffe 1965), whereas there is growing evidence that RHDV is a newly pathogenic variant of genus of generally non-pathogenic rabbit specific lagoviruses (Capucci et al. 1991; Cooke and Fenner 2002; Kerr et al. 2009). The two differ in transmission as well, with myxoma virus being transmitted primarily by arthropod vectors (Fenner and Ratcliffe 1965), while RHDV is transmitted primarily by direct contact between rabbits (Morisse et al. 1991), although arthropod vectors can spread it as well (Asgari et al. 1998; Cooke 2002).

One of the rabbit populations identified in Chapter Three to have a moderate level of resistance to RHDV is located at the Turretfield Research Centre in South Australia. A high proportion of rabbits from this site were resistant to infection, as determined by high survival and absence of seroconversion following experimental challenge. However, they were not resistant to lethal disease, as all rabbits from Turretfield that

did become infected died, albeit with unusually prolonged survival times (Chapter Three).

Additionally, since the release of RHDV in Turretfield in 1996, on-going monitoring using regular capture and recapture of rabbits has followed the population through 11 RHD outbreaks (Peacock and Sinclair 2009). Most importantly, virus samples were collected from rabbits found dead during each RHD outbreak over the 16 year period and stored in laboratory freezers. The archived samples now provide an excellent opportunity to study virus host co-evolution of RHDV. Sequence analysis is showing how the genetic make-up of RHDV has changed over time and, most importantly, virus samples from different years can be tested experimentally in naive progeny of today's genetically resistant rabbits from the site, to further compare the virulence of each virus isolate in resistant rabbit phenotypes (Dr R. Sinclair pers. comm).

In this paper, the relative virulence of the Czech 351 variant originally released in Australia and three naturally circulating isolates of Czech 351-derived virus collected in recent RHD outbreaks at Turretfield is examined in purpose-bred progeny of wild, genetically resistant rabbits from the same locality. We measured virulence as the effect the virus had on the rabbit in terms of mortality and survival times of rabbits that died as well as the virus titres produced in their livers. We demonstrate that RHDV is maintaining measurably high levels of virulence that appears to off-set developing genetic resistance in Australian wild rabbits.

Materials and methods

Experimental design

To ask whether the virulence of field strains of RHDV are changing, field strain viruses were obtained from the Turretfield field station (34° 33’ 00” S, 138° 49’ 47” E) from three outbreaks (2006, 2007 and 2009) for comparison with the original Czech virus released in 1995. These were tested in resistant laboratory-bred rabbits from the same site. The Turretfield field site is an agricultural research station approximately 50 km north of Adelaide, South Australia. It is in a cereal cropping, livestock grazing and grape growing area with a Mediterranean climate and average annual rainfall is around 470 mm. A detailed description of the site is given in Peacock and Sinclair (2009). The rabbits on the 12 ha study site mostly live in permanent warrens and the population is relatively isolated from other rabbit populations by cropping and grazing lands although there is some evidence of low level emigration and immigration on the site.

The site is regularly searched for rabbit carcases or sign of rabbits dying in warrens as indicated by blowflies (*Calliphoridae* and *Sarcophagidae*), their maggots or meat-ants (*Iridomyrmex* sp.) emerging from burrows or the smell of decaying carcases in and around warrens. Liver, heart, kidney or bone marrow from large leg bones are sampled from any carcase found and frozen at -30° C as soon as possible prior to the presence of RHDV being confirmed by RT-PCR followed by amplification of the PCR products for sequencing of either the partial capsid protein RNA or the entire

virus genome in selected samples (performed by Dr. D. Peacock, Dr. R. Sinclair and J. Kovaliski – Biosecurity SA).

Preparation of virus isolates for challenge tests

The field isolates came from three livers of individual rabbits found freshly dead and verified by PCR as having died from RHD. These were collected during winter/spring outbreaks of RHDV in 2006, 2007 and 2009 (TUR06, TUR07 and TUR09). The inoculum to which they were compared was a commercially available inoculum made from the original Czech CAPM 351 (Czech351) virus.

To standardise a dose for each of the four isolates, given their different origins, preparations and storage times, each was passaged through a domestic rabbit and, upon death, livers were stored and subsequently used to produce inoculums. Equal-sized (1 cm x 1 cm) sections of liver were removed and macerated in 5 mL of PBS and made up into equal volumes in PBS (50 mL). These stocks were then used to challenge rabbits. Subsequently, quantitative PCR (q-RTPCR) was used to obtain the number of genome copies per mL for each of the inoculums to confirm that similar doses had been administered (performed by Dr T. Strive, CSIRO).

Preparation of wild rabbits for challenge

Eleven rabbits (four males and seven females) were live trapped from the Turretfield site within on month of the 2007 RHD outbreak. These were housed at the Robert Wicks Pest Animal Research Centre, Inglewood, Queensland. Four females (seronegative to RHDV, to reduce the risk of young carrying temporary maternal antibodies) were randomly chosen and randomly paired to the four males (each

seropositive to RHDV) and housed in a temperature controlled animal house (20 ± 1°C, day/night cycle 12hr / 12hr) in large cages in an environment where freedom from exposure to RHDV and non-pathogenic RCV-A1 could be assured. Presence of his latter virus could have resulted in erroneous experimental results because it partially immunizes rabbits against the full effects of RHDV (Strive et al. 2009).

All rabbits were fed commercial pellets daily, carrots twice weekly and water *ad libitum*. From these four pairs, 80 offspring were reared and held until more than 13 weeks of age when infection and mortality rates are no longer influenced by age – related resilience (Robinson et al. 2002b). Prior to challenge, the young adults were assigned to four groups of 20, to distribute animals of each sex and known parentage equally.

For experimental challenge, rabbits were individually housed in plastic boxes (610 x 410 x 400 mm) with insect-proof gauze lids and wire mesh floors over absorbent litter. These were held in a climate-controlled room (22ºC ± 1ºC, 50% RH, 12hr / 12hr day/night cycle). Water and food (commercial rabbit pellets and fresh carrot) were provided *ad libitum*.

Monitoring and sampling of experimentally infected rabbits

Before challenge, a blood sample (0.5 mL) was taken from an ear of each rabbit to confirm freedom from exposure to RHDV and the non-pathogenic rabbit calicivirus RCV-A1. To initiate the experiment, the twenty rabbits in each group received 1 mL of the appropriate virus inoculums (i.e. Czech351, TUR06, TUR07 or TUR09)

administered orally using a tuberculin syringe (without needle) placed through the diastema onto the back of the tongue.

Following virus administration, rabbits were checked at eight hour intervals (nominally 0700, 1500 and 2300 hours) to minimise disturbance while still allowing time of death to be calculated and blood samples to be collected. Upon death, rectal temperature and a blood and liver sample were taken. Rectal temperature was used to calculate time of death since the previous check time (see Chapter Three for method). Serum samples were frozen and sent to Adelaide and tested by J. Kovaliski (Biosecurity SA) using a suite of ELISAs (Capucci et al, 1996) to confirm infection with RHDV. The liver samples were stored frozen at -20°C for four months before q-PCR assay. Rabbits surviving to 14 days post-administration were euthanised and blood and liver samples also taken for ELISA and q-PCR assay.

Statistical analysis

Mortality differences between the four isolates were measured using Chi-Squared analysis with Fisher's Exact (Sokal and Rolf, 1995) tests to determine which isolates differed significantly from the others. Survival times were analysed using analysis of variance and Tukey's multiple comparison test to find differences (Sokal and Rolf, 1995). As mortality and survival time are linked, Kaplan-Meier survival analysis (Kaplan and Meier, 1958) was used to further show differences between the four isolates. Differences in virus titres in deceased rabbits were tested using analysis of variance and t-tests.

Results

Experimental infections

The mortality rates (Table 6.1) differed significantly between the four strains of virus ($X^2 = 10.489$, d.f. = 3, p = 0.015), all animals infected with the TUR07 and TUR09 isolates died while 10 percent of rabbits infected with TUR06 and 25 percent in the group infected with the Czech351 isolate survived. The Czech strain caused significantly lower mortality compared with the TUR07 and TUR09 isolates (Fisher's Exact Test p = 0.0235 and p = 0.0235 respectively). Survival times of rabbits (Table 6.1) also differed significantly between the virus isolates (F = 7.66, d.f. = 3, p = 0.0002). The rabbits infected with Czech351 had significantly longer survival times than TUR07 (q = 6.596, d.f. = 69, p < 0.01) and TUR09 (q = 6.088, d.f. = 69, p < 0.01). Kaplan-Meier analysis similarly showed clear differences with TUR07 and TUR09 isolates having shorter survival times and higher mortality than the TUR06 and Czech351 isolates (Figure 6.1 A). Survival times for rabbits that died from each strain was closely associated ($y = -2.967x + 345.07$, $R^2 = 0.9917$) with the proportion of rabbits that died.

Table 6.1. Mortality rate and average survival times ± 95% confidence intervals and viral RNA copies per mg liver tissue (of rabbits that died) of rabbits infected by each virus isolate.

Virus Isolate	TUR06	TUR07	TUR09	Czech351
Mortality Rate	18/20	20/20	20/20	15/20
Average survival time (hrs) ±SE	81.17 ± 15.68 n = 18	44.45 ± 2.49 n = 20	50.35 ± 3.50 n = 20	121.27 ± 22.35 n = 15
Dose (genome copies total)	7.42×10^9	1.95×10^9	3.01×10^9	2.50×10^8
Average genome copies/ mg liver	4.74×10^8 n = 18	6.12×10^8 n = 20	1.12×10^9 n = 20	4.15×10^8 n = 15
STDEV genome copies/ mg liver	3.53×10^8	5.12×10^8	7.30×10^8	4.74×10^8

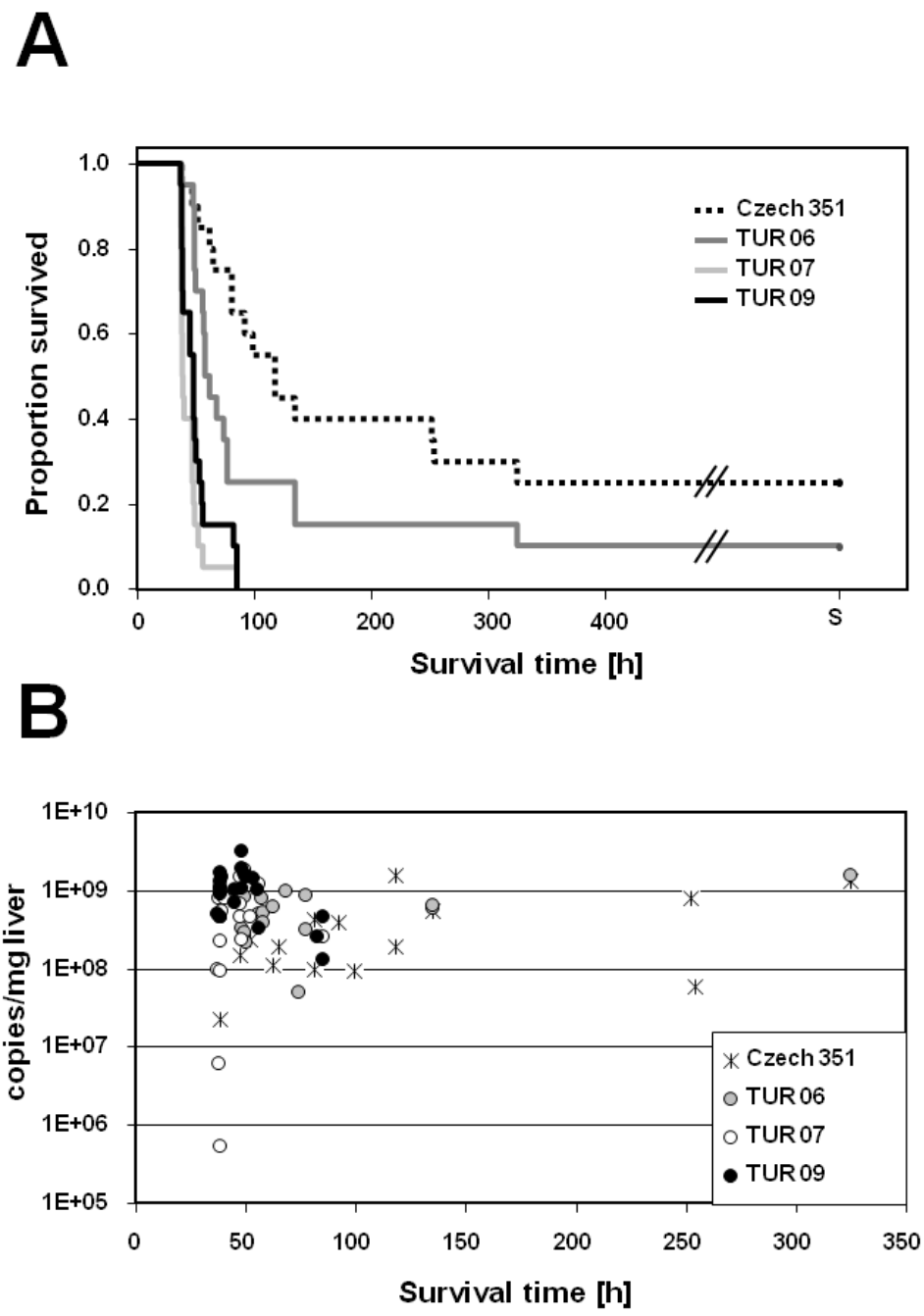

Figure 6.1: A: Kaplan Meier Survival analysis of rabbits infected with the different virus isolates. S: Surviving rabbits were euthanased 14 d.p.i. **B:** Viral RNA copy numbers in liver tissue and survival times in rabbits that died. Viral RNA copies are expressed as copies per mg tissue.

Quantitative real time analysis of the livers of dead rabbits showed that the three field-collected viruses had very different characteristics from each other and from the Czech 351 isolate. Figure 6.1 B shows the relationships between survival times and viral loads in the liver of dead animals. Rabbits infected with Czech 351 showing the longest survival times and also the lowest average viral loads (4.15×10^8). Infection with TUR06 resulted in comparatively shorter survival times and increased viral loads in the liver (4.74×10^8). Survival times in rabbits infected with TUR07 were even shorter, and average viral loads were comparable to TUR06 (6.12×10^8), however the variance in this group was high, suggesting that TUR07 killed some rabbits even before they had developed very high virus loads in the liver. In contrast, TUR09 killed rabbits equally quickly but had the highest titres of virus RNA in the liver. The mean RNA copy numbers per mg liver tissues in non-surviving rabbits varied significantly between treatment groups (table 6.1), (One Way ANOVA, $F = 5.81$, $P = 0.0013$), and the copy numbers of rabbits infected with the TUR09 isolate were significantly higher compared to all other treatment groups (t-test, p=0.002 TUR09 vs Czech351; p=0.006 TUR09 vs TUR06; p=0.02 TUR09 vs TUR07).

Quantitative PCR (qPCR) was used to determine the number of genome copies in the inocula used for challenge testing (Table 6.1). While not identical in terms of their genome equivalents, a very high infectious dose was delivered in each inoculums. Dose effects at such massive doses of infection are unlikely. This is further supported by the fact that TUR06 administered at the highest dose (copy number) showed many of the characteristics shown by Czech351 administered at the lowest dose in terms of copy number. Furthermore, it has been shown that the viral load in livers of rabbits

that succumb to RHDV infection is independent of the inoculation dose (Matthaei et al, in prep).

Seven animals survived the experimental infections, 5 in the group infected with the Czech 351 strain, and 2 in the TUR 06 group. Of these survivors, only one animal infected with the Czech 351 strain had seroconverted and produced antibodies against RHDV 14 days after the challenge. This indicated that the other rabbits had avoided infection, rather than resisting disease. Viral RNA was detected in the liver of these animals, albeit at very low levels (Table 6.2). Notably, initial seroconversion (IgM and a positive competition ELISA) was also found in two rabbits that did not survive the challenge, one of which was infected with the Czech virus and one with TUR 07. These animals had prolonged survival times that were amongst the highest in their group (135h and 85h, respectively, data not shown).

Table 6.2. Seroconversion and viral genome present in the livers of the seven rabbits that survived challenge with RHDV.

Rabbit ID	Group	seroconversion to RHDV	copies/g liver
168	Czech351	Yes	1.23×10^3
306	Czech351	No	1.36×10^3
314	Czech351	No	2.95×10^2
310	Czech351	No	1.78×10^2
353	Czech351	No	3.98×10^2
169	TUR06	No	6.50×10^3
336	TUR06	No	3.71×10^2

In summary, our results indicate that in genetically resistant rabbits, the relative virulence level of recent RHDV isolates increases in accordance with the year of isolation. Compared to the Czech strain initially released in 1996, field isolates collected in 2006, 2007 and 2009 show a general trend towards causing higher

mortality rates, shorter survival times and higher virus loads in the livers of rabbits succumbing to the disease (Figure 6.2).

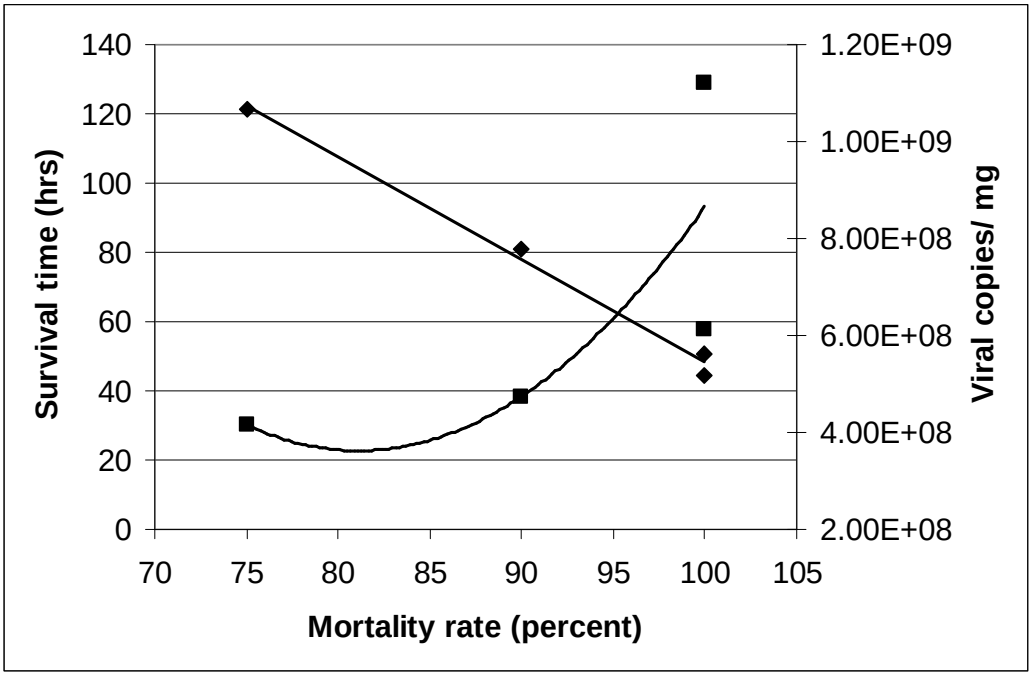

Figure 6.2: Evolutionary trends in RHDV virulence. Diamond symbols indicate average survival time of rabbits that died following infection with the different virus isolates. Square symbols show average viral loads in the livers of animals that died as a result of infection with the respective strains.

Discussion

It is apparent from the results presented here that TUR07 and TUR09 isolates show a higher virulence than the earlier isolates. This suggests that strains that maintain a high level of virulence have a selective advantage in the presence of increasing rabbit resistance. However, the results for TUR07 suggest that simply killing rabbits quickly may not be the best strategy. It is arguable that TUR07 may be at a selective disadvantage to TUR09 in resistant rabbits because it killed many without maximizing virus titres and chances of transmission. Likewise, if rabbit resistance is a factor underlying the extended survival of some rabbits then Czech351 and TUR06 are at a disadvantage because, even though rabbits survive for a long time and show relatively high virus titres, the virus may already be degraded and not transmissible. Indeed, once the rabbits' immune responses is activated, (and antibodies appear from about 3 days onwards see table above) the virus is quickly degraded into 'smooth-particles' which essentially lack the outer section of the capsid (Capucci et al. 1991; Granzow et al. 1996). In short, it appears that the virus should have the greatest chance of transmission and the greatest selective advantage if it produces the highest possible titres of viable intact virus. Viruses that kill the rabbit too quickly or too slowly are at a selective disadvantage.

The progression (Figure 6.1) seen with the more recent field strains having a more virulent phenotype in resistant rabbits than their antecedents may indicate that RHDV is evolving to match the resistance that rabbits developed to the Czech351 and TUR06 isolates. The similarity between TUR07 and TUR09 which killed all rabbits with short survival times may reflect the fact that challenged rabbits had only reached a

2006/2007 stage of resistance. If the work was repeated with today's rabbits, those viruses might show different, more distinguishable characteristics.

Recent models of virulence evolution are based on the idea that virulence can increase or decrease depending on the interactions between virulence, transmission and host recovery (May and Anderson 1983). In the example of myxoma virus, strains of intermediate virulence with reduced mortality rates and prolonged disease dominated due to a trade off between producing enough virus containing skin lesions for transmission without killing the rabbit before biting insects had enough opportunity to take up virus from these skin lesions. In contrast, the virulence evolution of RHDV appears to follow a different pattern. The data presented here shows that more recent field isolates cause higher mortality rates, death occurs more rapidly and higher viral loads are seen in the livers of dead animals.

The two viruses differ considerably in their biological properties. Myxoma virus is a large DNA virus (Fenner and Ratcliffe 1965) that interacts with the host's immune system at multiple levels (Kerr 2012). In contrast, RHDV is a small positive-stranded RNA virus (Ohlinger et al. 1990). Although the evolutionary rates of myxoma viruses are amongst the highest known for any DNA virus (Kerr et al. 2012), mutation rates in RDHV are several orders of magnitude higher, owing to the lack of proof reading ability of RNA virus polymerases (Holmes 2010; Hicks and Duffy 2012; Kerr 2012). In addition, the two pathogens are examples for two different emerging disease mechanisms. Myxoma virus causes only a mild fibroma in its natural host *Sylvilagus brasiliensis*, the tapeti or jungle rabbit, but became highly pathogenic when it crossed the species barrier and infected the European rabbit *Oryctilagus cuniculus* (Kerr

2012). On the other hand, there is growing evidence that RHDV is a pathogenic variant that has emerged by mutation within a genus of generally non-pathogenic, rabbit specific lagoviruses (Capucci et al. 1991; Cooke and Fenner 2002; Kerr et al. 2009). These non-pathogenic lagovirus pre-cursors have also been shown to have a different tissue tropism and cause a completely benign infection of the upper intestinal tract (Capucci et al. 1996; Strive et al. 2009; Strive et al. 2010; Le Gall-Reculé et al. 2011). On superficial consideration the evolution of virulence in RHDV might be seen as a case of 'short sighted' virulence evolution (Levin and Bull 1994), where a pathogen has a selective advantage within the same host by acquiring mutations that enable it to produce more offspring, invade different tissues and/or acquire mechanism to evade the immune system. Poliovirus is a frequently cited example for short sighted evolution as it sometimes acquires neuro-tropism and subsequent high levels of virulence, but does not gain an advantage from this beyond the current host as the new properties limit rather than increase transmission between hosts (Levin and Bull 1994). Similarly, RHDV has acquired the ability to replicate in the liver, replicate to extremely high virus titres and kills the host so quickly that it largely avoids adaptive immune responses. The important difference is that in the case of RHDV this evolutionary strategy is anything but short sighted, and the key to this likely lies in its main mode of transmission.

RHDV can be transmitted orally by direct contact between rabbits (Morisse et al. 1991). In addition, whilst neither myxoma virus nor RHDV are arboviruses, they can both be passively transmitted via insect vectors, and it was such insect transmission that led to the escape of RHDV from Quarantine in 1995 (Fenner and Fantini 1999). Myxoma virus is transmitted by biting or blood sucking arthropod vectors such as

fleas and mosquitoes (Fenner and Ratcliffe, 1965) that take up virus with their mouth parts and pass it on when visiting the next host for a blood meal. In contrast, distance transmission of RHDV occurs via flies that scavenge on decomposing carcasses, or fresh carcasses opened by predators and then transmit the virus passively by landing on mucous membranes of rabbits directly, or by leaving faeces or regurgita on pasture that is subsequently ingested by rabbits (Asgari et al. 1998; Cooke 2002, Barrat et al, 1998). The key difference is that myxoma virus vectors require a live, diseased animal, whereas for RHDV the carcass of a dead rabbit is the main source of virus for transmission.

While this may explain the selection towards maintaining extreme virulence, it raises the question as to how such high mortality rates can be tolerated by the system and not result in depletion of susceptible hosts and in turn negatively affect long term virus survival. The answer to this lies in another unique property of RHDV. Young rabbits can become infected with RHDV but are innately resistant to lethal disease. The exact mechanisms for this are not known, but lack of receptors or immaturity of the immune system have been suggested (Ruvoen-Clouet et al. 2000). This resistance is almost 100% but is gradually lost until the rabbits become fully susceptible at 12 weeks of age. Maternal antibodies can prolong the period of disease resistance but are not essential in this are-related resistance (Robinson et al. 2002b). Importantly, young rabbits survive the infection with a strong adaptive immune response providing lifelong immunity to RHDV (Cooke and Fenner 2002). These animals are then likely to be recruited into the immune breeding population, and the offspring they produce form the next generation of susceptible hosts. Infection during the early part of the life of the host can therefore explain how mortality rates of >90% can be tolerated.

Of the seven rabbits surviving the challenge infections in this study, only one seroconverted, suggesting that the mechanism of resistance is sometimes expressed as avoidance of infection, not simply resistance to lethal disease. Nystrom et al. (2011) suggested histo-blood group antigens (HBGAs) as co-factors facilitating the uptake of RHDV. Rabbits express a variety of different HBGA phenotypes on their intestinal tissues that differ in their ability to bind different RHDV, leading to resistance to lethal disease. These protective effects were only observed at low virus doses, and were overcome when high challenge doses were used. Chapter Three also demonstrated resistance of the Turretfield rabbits to infection with low doses of RHDV, although the HBGA phenotypes of the Turretfield rabbits used in this experiment were not known. The results suggest however that the putative genetic resistance mechanism in the Turretfield rabbits may also lead to less efficient uptake of virus, thereby effectively increasing the dose needed to infect a rabbit. In turn, it appears feasible that selection for virus strains with increased virulence that replicate to very high titres in the liver could partially off-set such resistance mechanisms. The amount of virus that is passively transmitted by flies that have fed on rabbit carcasses is likely to be very small. Consequently, maintaining high virus loads in rabbits that die from RHDV, before the onset of any adaptive immune responses, and causing mortality rates close to 100% would maximise the persistence of virus strains in the field. In this context the increased survival times caused by the earlier isolates Czech 351 and TUR06 in the resistant 2007 rabbits should also be noted – they may be a result of an effective reduction in dose due to resistance mechanisms at the virus entry level leading to more rounds of virus replication and thus a prolonged survival time. On the other hand, all surviving rabbits had low levels of RHDV- RNA in their livers.

While this may represent contamination at the time of sampling (rabbits were autopsied in the room where others had previously died from RHDV), it is also possible that this RNA represents virus inoculums taken up and transported to the liver that failed to establish a productive infection. This raises the possibility of a different or additional mechanism for avoiding infection and disease.

As previously mentioned, myxoma virus provides an example of an emergent disease that appeared by jumping from one species to another (Kerr 2012) while RHDV has apparently emerged as a pathogenic form of a previously non-pathogenic group of lagoviruses (Capucci et al. 1991; Cooke and Fenner 2002; Kerr et al. 2009). Together, they provide examples of the two main methods of disease emergence. The myxoma virus model provides insights into how the virulence of a large DNA virus may evolve when it jumps species into a novel host (Kerr 2012). By contrast, RHDV shows how a small pathogenic RNA virus that has emerged from a previously non-pathogenic group of viruses is apparently progressing along a different path and maintaining high virulence despite infecting the same host species, the European rabbit.

The situation in Australia provides a remarkable model of a host with three viruses representing three virulence mechanisms; RHDV maintaining a high level of virulence, myxoma virus an intermediate level of virulence (Fenner 1983; Kerr 2012) and RCV-A1 with an absence of virulence yet highly transmissible (Strive et al 2010). Further investigation of RHDV virulence evolution and epidemiological patterns over the next few years will provide insights into not only the RHDV-rabbit situation but co-evolution of viruses and hosts in general.

CHAPTER SEVEN

SYNOPSIS

There is strong evidence that the European rabbit in Australia is developing genetic resistance to infection with rabbit haemorrhagic disease virus. Evidence from challenge tests of groups of rabbits from many different localities across inland and south-eastern Australia effectively demonstrated this. As no resistance testing has been done in rabbit populations in Australia since the release of the virus, a spatial experimental design was used to test for differences in response between populations. This approach is based on the idea that resistance is likely to develop at different rates in different areas (Gandon 2002; Woolhouse et al. 2002) and my demonstration that rabbits from different populations showed different levels of response supports that view. Additional evidence of a geographical pattern to levels of resistance, also adds weight to the conclusions. Finally, it was found that these results were generally consistent with studies of RHDV infection that demonstrated the involvement of histo-blood group antigens (HBGAs) in virus binding and the outcome of disease (Nyström et al. 2011). As some of that work had been done in conjunction with Australian scientists and involved analysis of rabbit tissues collected in Australia, a genetic basis and potential mechanism to explain resistance is now apparent.

To consider the broad outcomes in more detail, discussion is made on the most relevant aspects in terms of: the usefulness and confirmation of spatial experiments for assessing resistance, population differences in resistance in Australia, including a speculation on interactions between RHDV and RCV-A1, the possible mechanism of resistance and the questions that resistance to infection raise, virulence evolution in RHDV, and comparisons to resistance and virulence patterns in myxoma virus.

Finally, to conclude, a discussion is made on the implications of resistance and virulence evolution for rabbit management.

Using spatial experiments to investigate resistance

Spatial patterns can be used to investigate resistance as they assume that spatially different populations are at different stages of the evolutionary process (Woolhouse et al. 2002). Spatial variation therefore can be used to infer temporal variation (Gandon 2002). It is important to establish that the difference has a genetic basis and is not simply the result of genetic drift in the resistance genes in the host (Woolhouse et al. 2002).

Populations that are spatially separate are under different environmental conditions and environmental factors certainly influence virus activity and host behaviours. For example, myxoma virus is most active in spring and summer in Australia, while RHDV is most active through winter and spring (Mutze et al. 2002). Rabbit breeding is resource and temperature dependent (Cooke 1974; Myers and Parker 1975; Cooke 1977; Tablado et al. 2009) and therefore breeding times can vary between sites which in turn may lead to young rabbits coming into the population and becoming susceptible (losing maternal antibodies) at different times of the year when RHDV may be more or less active. These factors can influence how resistance might evolve.

The differences in infection rates by RHDV between the spatially different populations in Australia indicate that resistance has developed. The population differences that may influence resistance were discussed in Chapter Three and are

elaborated upon below. The exact genetic basis for the resistance is still being explored, but phenotypic differences have been shown to determine survival against challenge with RHDV (see below). The use of spatial experiments to determine resistance against RHDV provided an alternative to long term studies, now impossible owing to the lack of resistance testing in the early years after RHDV was released.

Population differences and resistance to RHDV infection

The levels of infection seen in different populations of rabbits on challenge with low virus doses varied widely from no infections at Ingliston, near Bacchus Marsh in Victoria to 73% at Bulloo Downs in Queensland (Chapter Three), a similar rate to that seen in unselected domestic rabbits used as controls. This indicates that some populations show very little or no resistance while others have developed high resistance to infection with low doses of the original CAPM 351 Czech strain that was released in 1996.

As discussed in Chapter Three, it was expected that the presence of non-pathogenic lagoviruses may cause resistance to develop less quickly in cooler, wetter sites. The presence of the non-pathogenic virus RCV-A1 that affords some protection against RHDV complicates the co-evolution between rabbits and RHDV. If a rabbit has active antibodies against RCV-A1 when it is exposed to RHDV, it may survive when otherwise it would have died. This may allow otherwise susceptible rabbits to breed and keep their genetic type in the population. This will slow the overall rate at which evolution of resistance can develop.

Rabbit populations from regions of higher rainfall in Australia (which are often associated with being cooler) had lower resistance levels than populations with intermediate rainfall (Chapter Three). Early analysis of the efficacy of RHDV showed that its efficacy declined along a hot, dry – cool, wet gradient (Henzell et al. 2002). Controlled laboratory trials showed that ambient temperature did not have an effect on the pathogenesis of RHDV (Cooke and Berman 2000) indicating that other factors are responsible for lower efficacy in cooler regions, and the presence of RCV-A1 could be the explanation. RCV-A1 has been isolated from the populations at Valpine, Michelago and Bendigo (Jahnke et al. 2010) where rabbits showed moderate to low resistance. Ingliston, near Bacchus Marsh in Victoria had the highest resistance level and while RCV-A1 has been isolated from Bacchus Marsh (Jahnke et al. 2010), serological evidence shows it is present at lower levels than nearby Bendigo (Mutze et al. 2010a). Rabbits from Yambuk, a high rainfall site on the south-west coast of Victoria, showed the lowest resistance of all non-arid sites, but samples from this site have not yet been tested for the presence of a non-pathogenic lagovirus (Dr. Tanja Strive, personal communication). The level of activity of RCV-A1 may interfere with the activity of RHDV and the timing of outbreaks in populations from high rainfall areas and retard the development of resistance.

The lower resistance level seen in the populations from more arid areas (Chapter Three) was harder to explain. One possible explanation for this unexpected finding is that in populations such as Bulloo Downs, Alice Springs and Yanyanna it may be that RHDV infection is not the driving factor in rabbit survival and reproduction. In his considerations of selection for resistance to myxomatosis, Rendel (1971) suggested that where disease caused very high mortality, so few recovered rabbits were recruited

that they contributed little to the genetic make-up of the adult population. This is clearly possible in the case of RHDV as well because at Gum Creek in semi-arid South Australia (Cooke et al. 2002; Cooke unpublished observations) only three young rabbits were recruited into the adult population at the end of the 1997 breeding season and they represented only 8% of the total breeding population (all seropositive rabbits). That meant that the majority of young born the following year would have been produced by rabbits that had survived the initial RHDV outbreak in 1995 and those recruited in 1996; the few 1997-born rabbits would have contributed a relatively small increment in resistance to the next generation. This trend continued with only three rabbits born in 1998 and two rabbits from 1999 joining the breeding population, although they formed progressively higher proportions of the breeding population because the numbers of older rabbits fell due to natural mortality. So while resistant individuals will gradually form a high proportion of the breeding population, it will slow the rate at which resistance can develop. Additionally, if RHDV is not active for a period of time rabbits that are born may reach the breeding population without being exposed, thereby diluting the resistant gene pool. Rabbits from Yanyanna, near Gum Creek, now show moderate resistance to RHDV implying that poor recruitment of young into the breeding population is likely to have slowed the rate of increase in resistance rather than suppressing it completely.

Poor recruitment as a factor influencing the rate of development of resistance may need further investigation because it is difficult to explain on the basis of disease impact alone. It is generally thought that some young rabbits should survive RHD because they are infected when young and are naturally more resilient to disease than adults or because they have added protection from maternal antibodies (Robinson et

al. 2002b). However, it is worth noting that the 15 years following the introduction of RHDV were characterized by average to below average rainfall and often severe drought across much of southern Australia. This would have meant that rabbit breeding was limited and the production of young was naturally low. Survival of young would have been poor even without added mortality from RHD and myxomatosis.

The Bulloo Downs population appeared to be no more resistant to RHDV than the unselected domestic rabbits, suggesting that there had been very little natural selection for resistance. However, this may be an exceptional case. The population had not only been subjected to drought from 2000 - 2005 but also extensive warren destruction had been carried out to remove most rabbits (Berman et al. 2011). Few rabbits remained on the site and young rabbits made up only a small proportion (5% - 16%) of the population (Elsworth unpublished observations). While trapping to collect rabbits from Bulloo Downs for the challenge trials in Chapters Three and Five, it was found that all rabbits with antibodies to RHDV were over two years old, indicating that natural RHD outbreaks occurred only sporadically. RHDV spreads more slowly where rabbit population density is low (Mutze et al. 2010a) and so the disease may have visited this small isolated population at Bulloo Downs only rarely.

An alternative view on this was subsequently raised by my experiment to see whether I could artificially select for resistance in rabbits from the Bulloo Downs population. Given that some rabbits showed resistance and that strong selection rapidly produced a generation of rabbits resistant to low doses of virus ($60ID_{50}$) the question is raised: Why is this population no more resistant to infection than unselected domestic

rabbits? This is clearly a subject for further investigation as it raises the idea that in very arid, marginal habitats the cost of selection may be high relative to other opposing selective forces. This is discussed in more detail later.

A speculation on interactions between RHDV and RCV-A1

Rabbits from cooler-wetter regions in Australia had lower levels of resistance and I suggest that the presence of the non-pathogenic RCV-A1 may play a major in slowing the resistance process. The presence of a non-pathogenic lagovirus that affords protection against RHDV will have impacts on the virus itself. If the non-pathogenic virus is highly active at a site, then RHDV needs some way to remain infective in populations that are partially protected. It may be that sufficient numbers of rabbits in the population do not get this protection, or that infection after antibodies to RCV-A1 decline is sufficient for RHDV to persist. There has been serological evidence (albeit in relatively low densities) of non-pathogenic lagoviruses from the Flinders Ranges in South Australia prior to the release of RHDV and up to 2003 (Cooke et al. 2002; Mutze et al. 2010a), however recent serological tests from this region, including the rabbits captured from Yanyanna for experiments for this thesis, have not shown evidence of it still being present. It may be that RHDV can out-compete the non-pathogenic lagovirus under arid conditions and has eliminated the non-pathogenic virus from this site.

RCV-A1 has thus far only been isolated in populations with a cool-wet climate (Jahnke et al. 2010) and serological evidence of non-pathological lagoviruses has shown higher rates in cooler-wetter areas than arid regions (Cooke et al. 2002; Mutze

et al. 2008; McPhee et al. 2009; Mutze et al. 2010b). Similarly, non-pathogenic lagoviruses that infect rabbits have been detected in areas of high rainfall in Europe (Capucci et al. 1997; Trout et al. 1997; White et al. 2004) and New Zealand (O'Keefe et al. 1999; Parkes et al. 2002). This may be an indication that non-pathogenic lagoviruses are less suited to arid regions and may not be as successful or able to compete against more virulent virus under these conditions.

The mechanism of resistance

As the resistance to RHDV detected in rabbits is resistance to infection rather amelioration to disease severity (Chapter Three), this raised the questions of how this might occur and what the selective advantage is to rabbits. Resistance in rabbits to myxoma virus infection results in a less severe disease and allows the rabbits immune system to overcome the disease (Fenner and Fantini 1999). This has obvious selective advantage as the individual survives disease and builds an antibody response that will prevent re-infection. As most adult rabbits have antibodies against RHDV (McPhee et al. 2009) and high doses of virus can still infect most rabbits (Chapter Four), it would appear that resistance to low doses simply delays a rabbit becoming infected. Young rabbits are better able to survive RHD, suffering less liver damage and they are protected by maternal antibodies (Robinson et al. 2002b). From this, it seems a delay in infection would be a selective disadvantage as older rabbits are more likely to succumb to acute infection than are young rabbits. A possible explanation is a dual mechanism of infection whereby there is a greater disadvantage to the rabbit in being infected by a viral-facilitated route that leads to rapid acute disease compared to an as yet unknown second pathway requiring a higher dose of virus.

An understanding of the underlying mechanism of resistance would help in determining what the selective advantage of resistance to infection by low doses of RHDV might be. Recent research in Europe has begun exploring these questions, but as yet the exact mechanism is unknown (see Chapter Two). The analysis of Nyström (2011) showed that there was higher survival in A-B- phenotype rabbits challenged with low dose RHDV. It was also shown that G2 strains of RHDV's (to which the Czech 351 strain introduced into Australia belongs) do not bind to A antigens and only bind to B and H-type antigens and due to the nature of A antigens masking H-type antigens, G2 viruses do not bind well to A+B- phenotypes (Nyström et al. 2011). The A-B- phenotype is the most common phenotype seen in rabbits at Bacchus Marsh whereas the A+B+ phenotype is most common at Bendigo and Hattah, although Hattah had a significantly higher proportion of A+B- rabbits (Nyström et al. 2011). The Bacchus Marsh area (Ingliston) had the lowest infection rate with no rabbits being infected in direct challenge trials with a low dose (10^6 genome copies per mL) and the Hattah population also showed very low infection while Bendigo (Spring Hill) had a high infection rate (Chapter Three). This supports the suggestion that the Czech strain RHDV in Australia does not bind well to A+B- and A-B- phenotypes at low doses and this may be a key indicator of resistance in rabbit populations.

From the Bulloo Downs population, a generation showing complete resistance to infection with low doses of virus was reached very quickly through forced selection in which breeding was conducted from resistant rabbits only (Chapter Five). This suggests that there may be relatively few genes for resistance to infection with Czech RHDV and that these may be readily inherited by offspring. It may be that the

selective breeding resulted in all offspring having a phenotype that prevents binding of low doses of RHDV. However, the Bulloo Downs population showed high levels of infection and mortality in the initial challenge trials indicating very low levels of resistance (Chapter Three). The Bulloo Downs population may have a wide genetic diversity of resistant genes but only a small proportion expresses the resistant phenotype. This further suggests that RHDV may not be very active at this site and placing heavy selection for resistance. RHDV spreads slowly in low density populations (Mutze et al. 2010a) and this population has been reduced by 98% by warren ripping drought refuge (Berman et al. 2011). It could also mean that building resistance to RHDV may come at a cost to survival in this arid site that has been under the pressure of severe drought (Berman et al. 2011). Costs of resistance can be resource dependent (Boots 2011) and in the presence of fewer resources as a result of drought and possible lower virus activity owing to low rabbit numbers, non-resistant individuals may have a selective advantage (Chapter Five).

The dose – level effect seen by Nyström (2011) whereby differences in phenotypes determined survival at low doses but not high doses was mirrored in the results of wild rabbits showing increased survival compared to unselected domestics with the difference disappearing at high doses (Chapter Four). A viral facilitated pathway of infection would lead to quick disease development and damage to the liver and death. Resistance to this pathway by limiting virus binding to ABH antigens and preventing infection may allow the rabbit to mount a more controlled immune response via a second pathway.

Evolution of RHDV virulence

The establishment of resistance in rabbit populations in Australia does not automatically mean RHDV is becoming less effective as a biocontrol agent for managing rabbits. The evolution of the virus in response to the rabbit's resistance also needs to be considered. Current field strains of RHDV from Turretfield in South Australia appear to be maintaining a high level of virulence and can infect and kill a higher proportion of rabbits from that population than the Czech strain originally released (Chapter Six). This may explain why regular epizootic outbreaks at this site seem to contain the rabbit population which has not greatly increased over time (Dr. Ron Sinclair, personal communication). This suggests that field strains of RHDV are capable of matching resistance with increased virulence such that, while the original release strain may no longer be as effective in controlling rabbit populations, the field strains maintain greater virulence. Increases in rabbit numbers in some populations (Sandell 2006; McPhee and Butler 2010), may indicate that RHDV at those sites has not yet evolved to counter the higher virulence level seen at the Turretfield site, or the rabbit resistance may be slowly out-stripping the capacity of the virus to maintain high mortality.

The trade-off theory of virulence evolution (see Chapter Two) links virulence to transmission factors. Transmission of RHDV is primarily via the oral route through direct contact of excretions and secretions between rabbits (Morisse et al. 1991). For virulence to increase or remain high there needs to be a positive relationship between virulence and transmission (Alizon et al. 2008). To maximise transmission, RHDV replication needs to be high to produce as many viral particles as possible to pass into

secretions and excretions. RHDV replication occurs in liver hepatocytes causing hepatic necrosis (Park et al. 1995), which leads to death if enough damage is caused to the liver. Therefore, increased viral replication will lead to death of the rabbit. If death of the rabbit decreases the chance for transmission then it could be expected that virulence may decrease to protect the host. However, the proximity that rabbits live to each other in warren systems and the grooming behaviours of rabbits (Williams et al. 1995) means direct contact with rabbits or their excretions (faeces and urine) probably occurs frequently. Infection with RHD normally results in death within 2 – 3 days, with normal behaviours and no signs of disease up to about 6 hours before death (Lenghaus et al. 1994). The final stages of disease tend to cause the rabbit to return to the warren and very few rabbits that die from RHD are found above ground. This means that susceptible rabbits are likely to come in contact with cadavers in the warren which can remain infective for up to two weeks (Westbury 1996). It would seem then, that death of the rabbit does not restrict transmission, allowing a positive correlation between virulence and transmission. Therefore, following the model of the trade-off theory, selection would favour strains of higher virulence to ensure a high replication of viral particles for transmission.

In his models for evolution of microparasites, Fouchet (2009) demonstrated that evolution of virulence of RHDV can be influenced by the host population. In a deterministic model, the host population has little influence on the virus evolution and the most virulent strain should always be selected. This model leads to local extinctions of the host and virus and is a less realistic model when compared to interactions between rabbits and RHDV. In a stochastic model however, the influences are more complex and different virulence strains can be selected for under

different spatial host conditions (Fouchet et al. 2009). In small isolated populations highly virulent strains will go extinct and so lesser virulent strains are favoured, however, higher virulence strains compete better within a denser, widespread population and would be favoured if frequent exchanges of virus occur between populations (Fouchet et al. 2009).

Another way in which virulence could be maintained would be a change in virus structure that allowed binding to A antigens. This would also allow infection to proceed more easily in rabbits with B- blood group phenotypes and therefore few binding sites for the G2 RHDV strain in Australia. This would in effect make rabbits with the A+B- phenotype susceptible to infection and disease despite their resistance to infection with the original Czech strain. In France, G2 strains have been replaced by G3-G6 strains which all bind to A antigens (Nyström et al. 2011).

Comparisons to myxoma virus

After the release of myxoma virus into Australia and Europe, innate resistance in rabbits and attenuated strains, leading to intermediate levels of virulence, of virus were quickly seen (see Chapter Two). The structural and behavioural differences between myxoma virus and RHDV suggested that resistance and virulence would develop differently (see Chapter Two). Resistance to RHDV is resistance to infection by the virus, not amelioration of disease as it is for myxoma virus. This has an immediate impact on virus survival. Myxoma virus is still infecting rabbits and has opportunity to replicate and be transmitted. Indeed, it appears that the virus is more effective at evading the rabbit immune response within the skin at the inoculation site

than in other tissues of resistant rabbits (Best and Kerr 2000). This may allow the virus to replicate and transmit from the skin without causing damage to other tissues. Infection is essential for viral replication and RHDV will not be able to do this in resistant individuals.

Field strains of RHDV are maintaining a high level of virulence (Chapter Six). This may be a consequence of resistance preventing infection to low doses as the virus changes to take advantage of even the smallest opportunity of antigen binding. The route of transmission also provides a fundamental difference between RHDV and myxoma virus and may select for higher levels of virulence in RHDV.

These differences impact on how RHDV and myxoma virus interact in wild rabbit populations. In South Australia, myxomatosis outbreaks traditionally occurred in late spring and summer when mosquito vectors were most prevalent, however this changed with outbreaks occurring in Autumn and the spring/summer outbreak being delayed after the arrival of RHDV (Mutze et al. 2002). Where myxomatosis and RHD outbreaks occurred simultaneously, RHD outcompeted myxomatosis as a result of the longer incubation time of myxomatosis (Mutze et al. 2002). The presence of both viruses in a population has the potential to interfere in the coevolutionary processes of each virus with rabbits. Rabbits that are resistant to RHDV may be killed by myxomatosis and vice versa removing resistant animals and slowing the overall development of resistance.

Conclusions

The effectiveness of RHDV for rabbit control in Australia may now be population specific and dependent on the current stage of the co-evolutionary process and the influence of external factors such as resource availability and the presence of non-pathogenic caliciviruses. Releases of the commercially available Czech 351 strain will not be effective in populations such as Ingliston and Hattah in Victoria, Whetstone in Queensland and Turretfield in South Australia where resistance levels are very high. At these sites, the endemic field strains that are evolving against the resistance will either already be better suited to remain effective or hopefully are moving in that direction. In those populations with high resistance levels, integrated control techniques such as baiting or warren ripping would provide a survival pressure that does not select for RHDV resistance. One of the difficulties now faced in rabbit management is determining which populations are resistant. Challenge testing is costly and time consuming, requiring specialised facilities. If a clear link can be shown between histoblood group phenotype and resistance level, then shot sampling could provide tissue for analysis of the AB phenotypic make up of a population and therefore an indication of its resistance level. For example, the high proportion of A-B- rabbits at Bacchus Marsh, and A+B- rabbits at Hattah suggests these populations are more resistant to RHDV than the mainly A+B+ population at Bendigo. Phenotypic analysis of the populations examined in Chapter Three would provide further supporting evidence for the basis of resistance to RHDV.

The examination of additional strains that could be released into Australia has been initiated and follows on from the determination that rabbits are developing resistance

to the Czech 351 strain of RHDV released in Australia. It will be important that these compliment the field strains in Australia. If rabbit populations that have developed resistance have a higher proportion of B- phenotype rabbits then a strain that binds to B antigens will be of little value. If a new RHDV strain is released into Australia it will provide a further opportunity to study the evolutionary interactions between rabbits and the biocontrol viruses. Where the new strain and field strains are both active, competition may occur with one strain becoming dominant either the new strain replacing the field strain or the field strain preventing the establishment of the new strain. Some caution is needed in assuming that new strains will dominate because over forty years of introductions of Lausanne strain myxoma virus did not result in it persisting in wild rabbit populations presumably because of competition with myxoma virus field strains derived from the standard laboratory strain (Saint et al. 2001; Kerr et al. 2003; Berman et al. 2006). Thus there will be a need to closely monitor the interactions between the virus strains and the rabbits to determine if these new releases are effective.

A deeper understanding of the complexities of the interactions between RHDV and rabbits will also help in maintaining rabbit populations in Europe. It is possible that resistance will develop slower in Europe as there are numerous strains which bind to different ABH antigens (Fouchet et al. 2009; Nyström et al. 2011). This would put greater pressures on rabbit populations as no one phenotype would provide protection from infection by all strains. A clear understanding of the processes involved would enable management programs to try and prevent new strains moving into populations to allow rabbit populations time to evolve against the strains to which they are currently exposed. Other control techniques employed by landholders to minimise

damage to agriculture, especially shooting, may need to be reduced as it may not only keep numbers low, but may also slow resistance development by removing resistant individuals from the breeding population. There has been a history of releasing rabbits in Spain into areas where numbers are low to boost numbers for hunting and provide prey for endangered predators (Calvete and Estrada 2004). If ABH phenotype does provide protection from infection, then translocating rabbits that have inappropriate phenotypes for the endemic strains of virus in a particular area may detract from the evolution of resistance.

It is clear that understanding how resistance, infection and virulence interact in a system can aid in managing that system to attain a desired outcome. Theoretical models of co-evolution provides a framework for experimental research to better understand a system. In return, the information gathered from experimental research can help refine these models. The myxoma virus–rabbit system provided information that allowed models to predict that intermediate levels of virulence would dominate (Anderson and May 1982; Dwyer et al. 1990). This then aided in decision making, with further experimental research, such that the practice of releasing higher grade virulence strains, particularly the Lausanne strain, was no longer considered useful (Berman et al. 2006). The resistance against RHDV found in rabbits appears to prevent infection at low doses of virus which in turn appears to result in the maintenance of relatively high virulence of RHDV. The manner in which RHDV replicates in the liver and transmits through contact of secretions and excretions indicates a positive relationship between transmission and virulence which should also select for strains with relatively high virulence (Alizon et al. 2008). In field populations of rabbits RHDV will continue to be an effective control tool, keeping

rabbit numbers low, where resistance has not developed due to the presence of non-pathogenic lagoviruses or environmental and resource limitations on recruitment, or where virus evolution has maintained a high level of virulence to overcome the resistance. Continued monitoring of the RHDV - rabbit system across a range of populations from different environmental regions will further our understanding of the co-evolutionary processes occurring and the management of Australia's worst pest species.

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