A REVIEW OF RABBIT HAEMORRHAGIC DISEASE IN AUSTRALIA

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A report prepared for Australian Wool Innovation and Meat and Livestock Australia on the current status of Rabbit Hemorrhagic Disease and its continuing effectiveness as a biological control agent. This literature review includes recently published and unpublished information from both Australia and overseas to enable the future use of RHD to be considered in the widest possible context.

Executive summary

The introduction of rabbit haemorrhagic disease virus in 1995 marked an important milestone in Australia's long battle with introduced rabbits. This review considers what is known about the virus as a biological control agent: from its molecular structure to its ability to survive in the environment and its capacity for infecting rabbits and causing acute disease. The impact of the virus on wild rabbit populations is strongly influenced by climatic factors such as rainfall and temperature, no doubt because these influence rabbit breeding and general population dynamics as well as the behaviour and abundance of insects that transmit the virus. Analytical models comparing epidemiology in temperate and semi-arid parts of Australia confirm that there are large differences in the timing, intensity and impact of disease spread. Epidemiological studies within Australia have been carefully reviewed and compared with similar studies from New Zealand and Europe. However, despite common patterns emerging, better predictive models are still needed to provide a regional overview and for planning Australia-wide strategies if, as new evidence suggests, rabbits are beginning to develop genetic resistance to infection with RHDV. In the short term it is important to establish whether the virus is also changing and maintaining its infectivity. If this is the case, RHD will remain a useful biological control agent well into the future although, like myxomatosis, may not maintain levels of rabbit control adequate to avoid all environmental and economic impacts. The introduction of RHDV brought high environmental, economic and social benefits, justifying its release, but land mangers now need to be aware that additional rabbit control effort is required to keep rabbits at the low levels seen in the last few years. Generally this is best achieved by using well-established methods such as poisoning and warren ripping to capitalize of the presence of RHD. Prospects for initiating new, effective outbreaks of the disease are limited because the virus circulates naturally and is widespread.

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Background

Australia's rabbit problem

Wild rabbits, *Oryctolagus cuniculus* (L.), arrived in Australia on Christmas Day 1859. Landed in Geelong from the brig "Lightning", they were taken to Thomas Austin's property at Barwon Park in south-western Victoria and carefully maintained with the aim of breeding sufficient numbers for hunting. The success of this project exceeded all expectations and within a very short time overabundant rabbits not only allowed good sport but were also spilling onto neighbouring properties and beyond. Consternation grew, but despite efforts to limit their spread within the colony, rabbits were soon out of control. Within 70 years rabbits had spread across the southern two-thirds of the Australian continent, linking up with other introductions of semi-domesticated rabbits and becoming a major pest of agriculture and the pastoral industry.

Over time, rabbit-proof fences and poisoning using arsenic, strychnine and other chemicals would be developed, as well as trapping, fumigation and digging out of warrens. However, only a few individual farmers persevered with control measures and were able to maintain rabbit-free properties. The rest simply bore the recurrent losses and tried to match control efforts to the problem as it fluctuated seasonally.

At the time, the main focus was the cost of rabbits to agriculture and the pastoral industry and little regard was given for the rabbit's impact on natural resources although Ratcliffe (1938) recognized that in damaging natural vegetation rabbits helped expose large areas of soil to erosion. Additionally, it was often hard to separate the impact of rabbits from changes wrought by the pastoral industry and overgrazing by sheep and cattle, especially in times of drought and economic down-turn (Pastoral Lands Commission 1898).

Although biological control agents including diseases such as chicken cholera had been considered and investigated since the late 1800s, it was not until 1950 that the disease 'myxomatosis' was shown to be highly effective in lowering the rabbit population. Its introduction had been slow and politically turbulent with strong advocates such as Dame Jean McNamara clashing with eminent scientists including Francis Ratcliffe and William Clunies-Ross from the Council for Scientific and Industrial Research (CSIR), whose experiments had given them little cause to believe that the virus would help solve the rabbit problem (Fenner and Fantini 1999).

As things turned out, however, myxomatosis not only dramatically reduced the rabbit problem in Australia but it also paved the way for more rabbit control research and a broader approach to wildlife management in general. The Wildlife Research Section came to take on the status of a full CSIRO Division and the discipline of wildlife population ecology gave enormous insight into rabbit population dynamics and an understanding of native wildlife populations that had previously been missing. Collaboration with other research groups, most notably Prof Frank Fenner's group in

the John Curtin School of Medical Research, also led to a remarkable understanding of the epidemiology of myxomatosis and the eventual co-evolution of the myxoma virus and the rabbit host.

The CSIRO Division of Wildlife Research made enormous contributions in terms of understanding rabbit biology, distribution and behaviour, as well as developing the most effective methods for using poisons such as monosodium fluoroacetate (compound '1080'). However, the fine details of application of control methods and their effective combination to deal with rabbits in specific habitats remained the task of State Government departments with responsibility for rabbit control.

Oddly enough, despite its broad wildlife research interests, CSIRO contributed only sporadically to the understanding of rabbits as an ecological problem in Australia (e.g. Leigh *et al* 1987). Nonetheless, Hall *et al* (1964) experimentally demonstrated that rabbits were a factor limiting regeneration of *Acacia aneura* at Koonamore vegetation Reserve, South Australia, and Crisp and Lange (1976), Lange and Purdie (1976), Lange and Graham (1983) and Crisp (1978) subsequently showed that rabbits reduced the ability of many arid zone Acacias to regenerate successfully. This picture was extended with observations on white cypress pine in southern Queensland (Johnston 1968), acacias in the Northern Territory (Foran *et al* 1985) and sheoaks *Allocasuarina verticilliata* in more temperate areas (Cooke 1987) through the efforts of universities and State Government organizations. Current research (Murdoch 2006, unpublished), is documenting regeneration of the pine-buloke woodlands of north-western Victoria following the decline in rabbits brought about by the introduction of Rabbit Haemorrhagic Disease.

Although myxomatosis had a profound effect on Australia's rabbit population, its initial severe impact did not last. The virus attenuated into less virulent strains and rabbits rapidly built up genetic resistance so that by the 1970s it killed only about 50% of rabbits that became infected. Rabbits were never likely to return to the numbers seen before 1950, but widespread use of poisons and other control methods were needed once again to guard against significant economic loss.

Sobey and Rendel (1971) of the CSIRO Division of Genetics took a lead in this area, arguing that the rabbit-specific flea, *Spilopsyllus cuniculi* (Dale), should be introduced, even though earlier attempts by A. L. Dyce to bring it into Australia had failed because the flea's complex life-cycle was not fully understood (Fenner and Fantini 1999). After Dyce's first attempts, work in Britain (Mead Briggs and Rudge 1960, Rothschild and Ford 1969) had subsequently shown that breeding by the fleas was closely attuned to the breeding of the rabbits themselves. Hormones associated with pregnancy in rabbits directed the development of the fleas' oocytes and pheromone-like substances in young rabbits also affected the fleas' mating and egg-laying behaviour.

With that new understanding of host-parasite interaction, European rabbit fleas were successfully introduced (Sobey and Conolly 1971) and once they became widely established there were clear indications of improved transmission of myxomatosis and enhancement of the effectiveness of the disease, particularly in arid areas where mosquito transmission was poor (Cooke 1983). Nevertheless, it also became obvious that these rabbit fleas were unable establish themselves in the arid regions of Australia

beyond the agricultural lands (Cooke and Skewes 1988; Cooke 1990a) and it was argued that, from among several species of fleas known to be present on rabbits in Spain, a new arid-adapted species might be introduced to facilitate virus spread in the inland.

Four species of rabbit-specific fleas in southern Spain were evaluated (Cooke 1990b; Cooke, 1999) and from among these *Xenopsylla cunicularis* Smit was chosen for use in Australia. After further testing in quarantine these fleas were bred in large numbers and widely released from 1991 onwards (Mutze 1996). However, it was during this work on flea ecology in Spain in 1988 that attention was drawn to the first outbreaks of a new disease among wild rabbits. Rabbit Haemorrhagic Disease, or RHD as it became known, eventually overshadowed the release of the fleas.

Over the time that rabbit fleas were being investigated another much larger project had also been developed. The concept was termed virally-vectored immuno-contraception, and involved making a genetically-modified myxoma virus that produced proteins associated with rabbit reproduction, such as the *zona pelucida* protein from the ova, or proteins from sperm (Robinson *et al* 1997). A rabbit infected with such a GM virus would not only form normal antibodies against the virus but would also be induced to form antibodies against its own eggs or sperm. By reducing the fertility of rabbits in this way it was hoped to create a new and relatively humane bio-control agent. Enormous progress was made in this new research area and the idea was shown to be theoretically possible, but the immune response of rabbits was not long-lasting enough to reduce fertility beyond the first one or two litters (Mackenzie *et al* 2006). Given the added difficulty of introducing the recombinant virus in an environment where highly adapted field strains of virus were commonplace and effected most naïve rabbits in their first year of life (Merchant *et al* 2003) it was judged inadequate to suppress an animal with such renowned fecundity as the rabbit where at least 80% of reproductive females would need to be sterilized to drive the population down (Twigg *et al* 2000; Williams *et al* 2007). The program was regarded as unlikely to succeed and abandoned at the end of 2005.

Despite this, the concept of immuno-contraception is re-visited from time to time and Hamilton *et al* (2005) suggested that other vectors such as the trypanosome *Trypanosoma nabiasi* might be investigated as possible GM vectors. This trypanosome is already present in Australian rabbits and infection lasts for up to six months potentially promoting a longer-lasting immune response than a viral infection.

History of discovery and spread of RHD

Rabbit Haemorrhagic Disease was first described among domestic rabbits in China in 1984. Nevertheless, it was not considered to have originated in that country because its introduction was linked to the arrival of stud angora rabbits from German Democratic Republic (Liu *et al* 1984). It spread quickly and killed countless thousands of domestic rabbits causing great economic loss, but there were understandable delays in identifying the causative agent and for quite some time it was thought to be a parvovirus. Equally difficult was the task of developing vaccines and organizing methods to control the disease. As a major international rabbit meat

supplier, China was not able to guard against the export of contaminated rabbit meat and RHD soon became spread widely throughout the world.

Despite its probable European origin, RHD was viewed, no doubt correctly, as a new disease when it first appeared in Italy in 1986 and killed millions of farmed rabbits (Cancellotti and Renzi 1991). As its presence in other European countries became known, work to identify and manage the disease expanded. The causative virus, which became known as RHDV, was quickly described and identified as a calicivirus (Ohlinger *et al* 1991). Effective animal health measures were put in place to limit its spread in commercial rabbitries and suitable vaccines were developed.

Inevitably, RHD became established within wild rabbit population in Europe causing high mortality and raising major concern among hunting organizations and conservation groups. In Spain and Portugal the decline in rabbits has been associated with a major decline of predators which depend heavily on rabbits. These include the Iberian Lynx, *Felis pardus*, and the Imperial Eagle, *Aquila adalberti*. It was the initial heavy impact of RHD on wild Spanish rabbits in arid Almería province that first raised the idea that RHDV might be a suitable biological control agent for use in the arid and Mediterranean-like climatic regions of Australia (Cooke 2002).

Further north in Europe RHD did not have the same impact on wild rabbit populations but nevertheless its occurrence in commercial rabbitries generated a great deal of economic concern and research interest especially in terms of the origin and evolution of the virus (LeGall *et al* 1998) and in explaining why the epidemiology of RHD changes so markedly between southern Europe and the north (White *et al* 2001, Forrester *et al* 2003, White *et al* 2004).

Introduction of RHDV to Australia

Czech strain v351 RHDV was brought into Australia by CSIRO Animal Health and maintained with high security precautions in the Australian Animal Health Laboratory at Geelong, Victoria. It was carefully assessed to determine its effects on wild-type rabbits of all ages. Potential vectors of the virus including fleas, flies and mosquitoes were investigated and groups of wild-caught rabbits were assessed to estimate likely mortality rates. Importantly, some 28 species of vertebrates (mammals, birds and reptiles) representing a cross-section of domestic livestock and native Australian fauna were tested to verify that RHD was specific to the European rabbit. Along with practical experience gained in Europe, the conclusion was reached that RHDV was unlikely to cause disease in any other species if used to control wild rabbits in Australia.

As the probability that the virus would be used as a biological control agent increased, there was considerable debate about the steps that should be taken. The decision to release the virus was not to be taken lightly and it was asked whether a disease should be released if there was no guarantee that it would be effective. On that basis, trials were begun on Wardang Island, 5 km off the coast of South Australia to evaluate the virus under field conditions typical of large parts of southern Australia. Wardang Island had previously been used for testing of myxomatosis in the 1930s and so seemed a logical choice.

It was planned to test the spread of the virus among natural groups of rabbits and so large natural rabbit warrens were enclosed in double rabbit-proof fences and these were further enclosed in a larger, double fenced compound, the high outer fence being electrified and built to keep out predators such as cats. Major precautions were taken to reduce the risk that RHD would spread, including treatment of mosquito breeding areas and trapping of flies. Personnel working in the experimental pens changed foot-wear, gloves and clothing and washed hands and face on crossing through each barrier fence in the compound. All rabbits experimentally infected were fitted with radio-transmitter neck collars and had reflective coloured ear tags so that they could be monitored using both radio signals and evening and night-time observations from elevated hides near each experimental pen. The frequency of the radio signals changed if the rabbit died and rabbits could be accurately located and retrieved even if they died below ground.

Nonetheless, despite precautions, the virus escaped from quarantine (Fenner and Fantini 1999) and became established on the mainland where it rapidly proved itself to be well adapted to the general environment. Within 18 months it had spread naturally over the southern half of the Australian continent (Kovaliski 1988).

Outcomes and current situation

RHD caused a reduction of over 90% in rabbit populations where it first established itself in arid inland areas (Bowen and Read 1998; Mutze *et al* 1998) and continuing outbreaks each year drove many populations even lower over the ensuing 5 years. However, results were not so spectacular in areas with cooler, more humid climates (Saunders *et al* 1999). At Cattai, near Sydney, there was no clear evidence that the virus was able to become established despite repeated releases (Richardson *et al* 2007). Nonetheless, it is widely considered that RHD generally reduced the Australian rabbit population by about 60%.

Despite the establishment of a national committee to oversee and make recommendations for continued monitoring of post-RHD rabbit populations, political interest was not maintained and by 2000 – 2001 few of the programs set up to monitor the disease were still supported financially. By 2005, there had been even further decline in interest despite rabbits remaining the most abundant introduced mammal species in Australia with the possible exception of the introduced house mouse (*Mus musculus*).

This situation raised concerns in Australian Wool Innovation and Meat and Livestock Australia representing major livestock industry groups. Australia was in a poor strategic position should RHD fail to keep rabbits low in the longer run. Underlying this was the concern that rabbits were likely to develop genetic immunity or RHDV might become attenuated. This was a likely outcome given that rabbits had become partially resistant to myxoma virus in the decade following its release. The questions asked included: If rabbits do begin to rise, what action should be taken to prevent this? What could be done to make the most of RHD at the present time? Are there adequate human resources and skills to manage the situation?

The industry-driven interest in rabbits proved to be timely. In the spring of 2006 a resurgence of rabbits was reported from many areas in north-western Victoria and South Australia. Moreover, many land managers had not been able to react effectively to counter increasing numbers. Much of the rabbit control work done on mallee roadsides by farmers trying to protect adjacent crops was hasty and barely effective. Like-wise, rabbit control efforts in Victoria's Hattah-Kulkyne National Park fell short in protecting regenerating native ecosystems despite considerable effort.

The *ad-hoc* responses to the upsurge in rabbits indicated that the infra-structure, skill-base and detailed knowledge built up during the 1970s and 1980s for controlling rabbits had been severely eroded. Changes in government policies, such as lower investment in agriculture-related research, lower investment in agricultural extension and advisory services and changes in the management of pesticides such as '1080', were partly to blame. Many people with practical experience in managing rabbit problems had also retired from the workforce over the intervening decade and there had been little incentive for maintaining training courses for replacement staff and advisors.

It was also clear that the benefits that accrued from the state government-supported programs such as "Bunny Buster" in Victoria, "Rangelands Action Program" in South Australia and "West 2000" in New South Wales had not been as fully publicized as deserved for their part in removing rabbits that remained in the wake of RHD and averting widespread resurgence of rabbits over relatively large areas.

Accepting recent evidence as indicative of a re-emerging rabbit problem, there are two pathways that might be followed. The first is to consider how RHD might be supported to maintain its usefulness; the second is to consider what alternatives may be available for maintaining rabbits close to the current levels. Any opportunities for taking rabbits lower, as advocated and partly implemented after the initial success of RHD, had been lost in all but a few areas due to the complacency of the last few years.

Objective of current review

This review is the first step towards asking how RHD might be supported and further exploited to wring the greatest benefits from it. It seeks to bring together published information on rabbit haemorrhagic disease from across the world, and match it with information from Australian studies to provide a strong base for making future management decisions. It considers evidence ranging from a basic understanding of the structure and function of RHDV through to questions relating to the development of genetic resistance in rabbits and finally considers practical suggestions for helping land-managers to guard against any wider resurgence of rabbits in the future.

Rabbit haemorrhagic disease virus

Classification

Rabbit haemorrhagic disease virus is a member of the family *Caliciviridae*. The name is derived from the Latin *calici* = cup referring to the regular cup-shaped depressions on the virus surface. It has further been classified as a Lagovirus along with the closely related European Brown Hare Syndrome Virus (EBHSV). *Lago* is derived from *Lagomorpha*, the rabbit family, which are hosts of these viruses. Lagoviruses are among four groups officially recognized within the *Caliciviridae*, the other genera are: *Vesivirus*, *Norovirus* and *Sapovirus*. The vesiviruses include San Miguel Sea-lion Virus (SMSV) and Feline Calicivirus (FCV) while type-specimens for the other genera are human Norwalk Virus (Norovirus) and Saporro Virus (Sapovirus) respectively (Green *et al* 2000).

Structure of the virus

In common with all other Caliciviruses, RHDV is a non-enveloped, positive-sense, single-stranded RNA virus. The genome contains 7437 nucleotides excluding the poly (A) tail arranged within two open reading frames (ORF1 and 2) of 2,344 and 118 codons respectively. In addition to the full-length genome there is a more abundant sub-genomic mRNA of approximately 2.2 kb containing the capsid gene (Meyers *et al* 1991; Wirblich *et al* 1996).

Within the single 60 Kda structural protein (VP60) the highly conserved S domain, a moderately conserved P1 sub-domain, and a hypervariable P2 sub-domain, provide for virus assembly, receptor recognition and evasion of the host's immune system. Viral particles are about 30 *nm* in diameter and have 32 cup-shaped depressions arranged in T = 3 icosahedral symmetry. Recombinant RHDV capsid protein expressed in baculovirus self-assembles into virus-like particles (Laurent *et al* 1994) although Barcena *et al* (2002) have shown that mutant coat protein lacking the normal N terminal sequence assembles into capsids with a different (T = 1) configuration.

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 their approximate molecular mass. Immunoblot analyses show that a minor structural protein of 10 kDa is encoded in ORF2.

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 by proteolytic cleavage of the large poly-protein (Sibilia *et al* 1995).

The polymerase in RHDV is an RNA-dependent RNA polymerase (RdRP). A 50 nucleotide promoter upstream from the start site for the sgRNA, at the 5' end of ORF2, is necessary for its regulation (Morales *et al* 2004). Vázquez *et al* (2004) have produced the polymerase of RHDV in *Escherichia coli* and demonstrated that it is enzymatically active. It generates the minus strand of RNA initiating the process at the 3'-terminal–OH.

The polymerase of RHDV was the first calicivirus RdRP to be studied in detail by x-ray crystallography (Ng *et al* 2002) and comparative studies with related viruses are advancing the understanding of the structure and operation of RdRPs in general (Bruen 2003).

Conserved motives in the genome often indicate the likely function of viral proteins and Marín *et al* (2000) correctly anticipated that p37 of RHDV should have ATP-binding and ATPase activity similar to the 2C protein of picorna viruses.

Recently there has been considerable interest in genome-linked viral proteins (VPg) of caliciviruses in general because they bind to major cellular proteins (e.g. elF3 and elF4E) and so facilitate virus protein translation (Draughenbaugh *et al* 2003). VPg is covalently linked to both the full length genome and the subgenomic mRNA.

Origin of RHDV

In Europe a number of viruses related to RHDV have now been described e.g. RCV (Capucci *et al* 1996), Rainham (Forrester *et al* 2006b). Additional variants of these apparently non-pathogenic lagoviruses are soon to be described from France (Jacques Le Pendu, INSERM, Nantes, pers. comm.) and Australia (Tanja Strive, CSIRO Entomology, pers. comm.) These viruses only share 60 – 70% similarity with RHDV and Fenner and Fantini (1999) have argued that these represent groups originating from the same precursors that gave rise to RHDV at some time in the past. The non-pathogenic Australian virus appears to be more distant from RHDV than its present day European counterparts (T. Strive, pers comm.), possibly arguing for long isolation and consequent divergence.

Host specificity

Despite concern that RHDV may not be specific to rabbits (Smith 1998), all information up to this stage points to the fact that conclusions drawn from challenge tests on 28 species of vertebrates in the Australian Animal Health Laboratory were correct. The European rabbit is the only host of RHDV. Even other lagomorphs that have been experimentally exposed show no clinical signs of disease; these include: the eastern cottontail (*Sylvilagus floridanus*), black-tailed jackrabbit (*Lepus californicus*), and volcano rabbit (*Romerolagus diazi*). The European brown hare (*Lepus europaeus*) and the varying hare (*Lepus timidus*) appear not to be natural hosts for RHD but are susceptible to the closely related EBHSV that causes European brown hare syndrome. Interestingly, the varying hare only becomes infected with EBHSV where its population overlaps with the European brown hare (Gavier-Widen and Morner 1993). Because EBHSV cannot maintain itself in populations of varying hares alone, the varying hare seems unlikely to be the true host.

Etherington *et al* (2006) have recently used available sequence data and phylogenetic analysis tools to consider the evolutionary paths of caliciviruses and their hosts and conclude that caliciviruses have occasionally switched hosts but there is no evidence that caliciviruses from any other mammalian groups have entered human populations as a result of zoonoses (disease spread from wildlife). Rather, host switching of caliciviruses, such as the case of San Miguel Sea-lion Virus (SMSV), has been associated with feeding sea-lion meat to swine (Smith *et al* 1973) and the close genetic similarity of some human, bovine and porcine caliciviruses suggests that switches in both directions may have occurred in association with the prehistoric domestication of livestock (Van Blerkom 2003).

There is insufficient evidence to precisely determine the age or demography of caliciviruses but one approach used by Etherington *et al* (2006) suggests that the present day *Lagoviridae* arose as a result of an ancient switch between carnivores and their (proto-) lagomorph prey about 60 million years ago during the Palaeocene period when mammals were radiating into ecological niches left by recently extinct dinosaurs.

Transmission

When RHD first spread in domestic rabbits it became clear that rabbit to rabbit contact and aerosol droplet transmission between closely-spaced cages were common means of transmission. Spread between rabbitries could usually be accounted for by movement of contaminated material on footwear, foodstuffs and cages (referred to as fomites) and by poor livestock hygiene measures among people handling stud rabbits or rabbits taken to abattoirs (Morrisse *et al* 1991). Gehrman and Kretzschmar (1991) nevertheless showed experimentally that stable flies of the genus *Phormia* were able to transmit the virus after feeding on the conjunctiva of rabbits. Only a few viral particles were required for infection by this route.

Experimentally, RHDV is known to be transmitted not only by oral, ocular and nasal routes but also by intra-dermal and sub-cutaneous injection as well as intra-muscular inoculation. Spread involving biting insects such as mosquitoes *Culex annulirostris* and rabbit fleas (*Spilopsyllus cuniculi* and *Xenopsylla cunicularis*) can be expected on the basis of laboratory experiments (Lenghaus *et al* 1994). It is also known that bush flies, *Musca vetustissima*, can transmit RHDV in the laboratory by feeding on the conjunctiva of infected rabbits (McColl *et al* 2002).

Nonetheless, scavenging flies such as *Calliphora* spp. are the insects most closely linked to natural RHD outbreaks. Using PCR, pools of trapped flies have widely been found positive for RHDV during disease outbreaks in both Australia and New Zealand (Asgari *et al* 1998; Barrett *et al* 1998). Asgari *et al* (1998) demonstrated the presence of virus in the faeces and crop regurgita in blow flies fed on infected rabbit livers. There were sufficient live virus particles in a single fly spot (regurgitated crop contents or faeces) to infect both domestic and wild rabbits.

Cell binding and replication

The known ability of RHDV to bind and clump human red blood cells (see also haemagglutination tests) led to the discovery that RHDV attached to histo-blood

group antigens on the surface of human erythrocytes and it was shown that similar antigen producing cells are distributed in the mucosal lining of the rabbit's gut (Ruvöen-Clouet *et al* 2000). Both native RHDV and virus-like particles produced in bacculovirus bind to synthetic A and H type 2 oligosaccharides.

This pioneering work was subsequently directed at Noroviruses (NV) and it was quickly shown that they too infect their human host using similar binding systems: AB, H and Lewis antigens are involved and secretors and non-secretors show different rates of infection (Huang *et al* 2005). Human blood group antigens are known to be complex carbohydrates linked to glycoproteins or glycolipids that are present on red blood cells and mucosal epithelial cells or as free antigens in biological fluids such as blood, saliva, intestinal contents and milk. Glycosyltransferases (e.g. fuctosyltransferase or FUT) controlled mainly by the ABH, Lewis, and secretor gene families are responsible for sequential additions of monosaccharides to the active portion of the antigen precursors (Le Pendu *et al* 2006).

Thorven *et al* (2005) have recently shown that a single mutation on the human secretor (*FUT2*) gene provided non-secretors with resistance to symptomatic infection during the natural spread of NV and Hutson *et al* (2005) have used samples from previous experiments using human volunteers infected with NV to show that resistance was linked to non-secretor status. Nonetheless, Rockx *et al* (2005) make it clear that not all strains of NV behave in the same way and there is no strong evidence of a genetic basis for resistance to some strains of NV.

Interestingly, Ruvoen-Clouet *et al* (2005) have shown that lipases and mucins in the milk of secretor mothers prevent the binding of Noroviruses and these may act as decoys to prevent infection of young. Secretors and non-secretors may therefore have advantages at different times and, if NV has had a long history of co-evolution with humans, this might be another factor contributing to the maintenance of stable histo-blood group polymorphisms in the European population.

It is known that rabbits have genes analogous to the secretor genes in humans (e.g. Hitoshi *et al* 1995) and investigations of the *Fut2* and *Sec1* genes are in progress in Europe (P. Esteves, P. Guillon pers comm). High polymorphism is apparent in the 2 genes, with 14 and 25 variable positions for *Fut2* and *Sec1* respectively.

Of more immediate practical interest is the knowledge that, as found for humans, the ABH antigens in rabbits require a considerable period for maturation (glycosylation). They become fully developed only when rabbits reach about 6 weeks of age and this has been suggested as an explanation for the natural resilience of young rabbits to RHDV infection (Ruvöen-Clouet *et al* 2000). Nevertheless, Ferriera *et al* (2006) have questioned the idea that an absence of receptors provides an adequate explanation pointing out that young rabbits inoculated intra-muscularly did not develop RHD but showed only a mild and transient hepatitis. These arguments are supported using evidence from electron microscopy to show that liver leukocyte infiltration in adults is associated with removal of dead hepatocytes whereas lymphocyte infiltration of younger rabbits is apparently associated with the expression of viral antigen on hepatocyte surfaces.

Interestingly, field epidemiological studies in semi-arid Australia suggest that RHD spreads most rapidly among mature but susceptible rabbits as they begin breeding. It would be worth investigating the possibility that RHD transmission may be enhanced by reproductive changes. In rats, the *Fut2* and *Sec1* genes are important for blastocyst adhesion to the uterine wall and *Fut2* mRNA is cyclically produced and shows a 10-fold change during oestrus (Domino *et al* 2001). If something similar occurs in the seasonally poly-oestrus rabbit this may enhance susceptibility among reproductive animals.

Pathogenesis

The disease caused by RHDV in adult rabbits is characterized by high morbidity and mortality rates and in domestic rabbitries in Europe losses of up to 90% were reported as the virus first spread. When RHD first spread in Australia, it was estimated that the morbidity and mortality rates of wild rabbits in the Flinders Ranges were 98% and 97% respectively (Mutze *et al* 1998).

The time from infection to death depends on the route of infection (Cooke and Berman 2000). Orally infected rabbits die about 60 hours after infection, about 21 hours later than those inoculated subcutaneously or intramuscularly. Infected rabbits become pyretic although body temperature may fall below normal in late stages of the disease (Robinson *et al* 2002) and death results from widespread circulatory dysfunction associated with disseminated vascular coagulation and necrotizing hepatitis. Rabbits appear to behave normally until about 12 hours before death and may continue to eat sporadically until a few hours or sometimes minutes before death. Just prior to death, there may be intermittent short struggles, rabbits lying on their side and paddling. This may be followed by a period in which the rabbit may right itself or continue lying in a comatose state until death. Bloody mucous discharge from the nose reported in domestic rabbits prior to death from RHD has not been reported among experimentally infected wild rabbits in Australia. Internally, the liver is pale and discoloured with a reticulate pattern, the spleen is swollen and there may be haemorrhagic lesions in the trachea, lungs and occasionally the kidneys.

Gender and body weight have some influence on survival time with female rabbits dying ahead of males and heavy rabbits dying before lighter ones. However, ambient temperatures between 13ºC and 27ºC do not appear to influence the course of the disease (Cooke and Berman 2000) even though high environmental temperatures can have a powerful effect in reducing mortality rates for myxoma virus infection (Marshall 1959).

Large quantities of virus are found in the rabbit's liver and other organs and virus particles are present in discharges from the nose. In adult rabbits, virus may also be excreted in urine and faeces commencing about 36 hours after sub-cutaneous infection. In young rabbits this is further delayed to about 48 hours (Shien *et al* 2000). Rabbits with acute RHD may shed virus for little more than 12 - 24 hours before dying whereas, from an epidemiological perspective, rabbits that recover from the disease may be more likely to spread virus in contaminated excreta.

At a more detailed cellular and biochemical level, programmed cell death or apoptosis of infected hepatocytes has been described (Alonso *et al* 1998, Jung *et al* 2000) and

the release of hepatocyte enzymes including aspartate aminotransferase and alanine aminotransferase into the blood has been used to monitor severity of infection (Ferreira *et al* 2006). San Miguel *et al* (2006) have shown that the administration of N-acetyl-cysteine reduces liver cell-death, probably by inhibiting the pathway of apoptosis.

Rabbits that recover from RHD appear normal although severe liver damage including loss of glycogen reserves (Ferreira *et al* 2006) may cause prolonged ill-health or death. There have been reports of 'earless rabbits', technically called chondropathy of the pinna, in association with outbreaks of RHD in colder regions of New Zealand (Clark *et al* 1999). It has been suggested that disseminated vascular coagulation in conjunction with low environmental temperatures might severely restrict peripheral circulation in the ears leading to tissue damage.

Diagnostic methods

A wide range of techniques have been used for investigating RHDV, studying its structure and following its epidemiology. These include electron microscopy, x-ray crystallography, in-situ hybridization, polymerase chain-reaction (PCR) and nested-PCR, nucleotide sequencing, and Western blotting. Detection of antibodies in rabbit sera initially involved haemagglutination techniques but these were abandoned when a wide array of enzyme-linked immuno-sorbent assays (ELISA) was subsequently developed (Capucci *et al* 1991).

For epidemiological studies in the field, ELISA techniques are particularly useful for detecting virus antigens and antibodies to them. Commonly, *virus capture* ELISA (vcELISA) is used to detect viral particles, and a *competition* ELISA (cELISA) is used to detect antibodies in rabbits that have recovered from infection with RHDV. Antibodies can be further analysed using *isotype* ELISAs to distinguish between different types of antibodies including IgA, IgG and IgM. The ratios of these isotypes give some insight into the antibody status of rabbits. For example, young rabbit kittens with exclusively IgG antibodies are almost certainly carrying antibodies of maternal origin. Likewise, young rabbits with high IgM titres are likely to have recently recovered from RHD (Capucci *et al* 1991, Cooke *et al* 2000).

Age-related resilience to RHD

In contrast to adult rabbits, kittens less than three weeks old do not develop disease when experimentally infected with RHDV (Morrisse *et al* 1991). Prieto *et al* (2000) have reported from immuno-histological studies that RHDV was not detectable in the livers of experimentally infected domestic rabbits less than 4 weeks old and only a few hepatocytes were involved in six week old rabbits. However, VP60 was detected as early as 12 hours post infection in hepatocytes of adults and reached a peak of about 60% of hepatocytes infected by 48 hours. Extra hepatic VP60 only appeared at 36 hours. These authors considered that hepatocytes were the only cells in the liver that supported RHDV replication.

A similar pattern is observed in wild rabbits. In experimentally infected nestlings (less than 3 weeks old) the virus fails to replicate in the liver beyond small foci (Lenghaus

et al 1994). As kittens become older, mortality increases reaching about 50% in six-week old kittens.

Importantly, antibodies of maternal origin passed across the placenta to late-stage embryos (Merad and Wild 1992) provide additional protection for up to 12 or 13 weeks of age depending on the mothers' antibody titres (Cooke *et al* 2000). Robinson *et al* (2002) experimentally quantified this for young Australian wild rabbits and demonstrated the outcome using analytical statistical models. By three months of age (12 weeks) young wild rabbits show the same mortality rates as adult adults. Maternal antibodies are most readily followed by measuring IgG isotype titres in young rabbits; however, these are not necessarily the main protective components because Robinson *et al* (2002) demonstrated that young rabbits were still protected from acute disease even though IgG antibodies could not be reliably quantified.

Maternal antibodies do not protect young rabbits from infection even though they help prevent development of disease. Tables 1a and 1b below (Cooke, unpublished) show details of a group of young wild rabbits captured for experimental purposes that subsequently became infected by RHDV while in transit to a laboratory animal house. The morbidity rate among these rabbits was unaffected by the presence of detectable maternal antibodies (reciprocal IgG antibody titre ≥1:40) but all rabbits with maternal antibodies survived infection whereas most unprotected young died. The mortality rate among the unprotected kittens was a little over 80%.

Table 1a. Young rabbits with and without maternal antibodies contracted RHD at similar rates (Cooke unpublished). (The G-test is a corrected χ^2 test)

	No antibodies	Maternal antibodies	Total
Not infected	10	4	14
Infected	13	6	19
Total	23	10	33

G = 0.03, p = not significant

Table 1b. Infected young rabbits with maternal antibodies had higher rates of survival than those without antibody protection.

	No antibodies	Maternal antibodies	Total
Survived	2	6	8
Died	11	0	11
Total	13	6	19

G = 13.4, $p < 0.001$

Immune response in rabbits that survive RHD

Rabbits that recover from RHD normally begin to develop antibodies within 4 days. An initial IgM response is followed within 2 – 3 days by a rise in IgA titres and a more prolonged IgG response. Nevertheless, in most rabbits titres of all antibody titres

decline with time: IgM antibodies usually disappear completely within 60 days although IgG antibodies usually persist at detectable levels throughout the rabbit's life.

Vaccines against RHD

Several RHD vaccines have been produced. Most are made from inactivated virus preparations derived from the livers of infected rabbits. Vaccines such as Cylap HVD® use a liquid oil adjuvant to promote antibody response. Recombinant RHDV capsid protein expressed in baculovirus and emulsified in Freund's complete adjuvant also gives protection against the development of RHD within five days of inoculation (Laurent *et al* 1994). Boga *et al* (1997) used the RHDV major capsid protein produced in the yeast *Saccharomyces* to induce protection without an adjuvant.

There have also been attempts to use recombinant VP60 capsid expressed in potatoes under the control of cauliflower mosaic virus 35S promoter as a vaccine against RHD (Castañón *et al* 1999). The product could be used to immunize rabbits if inoculated intramuscularly with a suitable adjuvant but the concentration of recombinant capsid protein was not high enough to immunize rabbits fed on the transgenic potatoes alone. Nevertheless, Gil *et al* (2006) have suggested that oral immunization might be achieved if different types of plants (e.g. *Arabidosis*) and other promoters were used to increase the concentration of VLPs. Such a claim should nevertheless be taken cautiously given that fact that rabbits exposed to low doses of live RHDV frequently avoid infection without developing antibodies (see *Resistance to RHDV infection*).

While vaccines are largely used to protect domestic rabbits from RHD, a matter of greater concern for Australia and New Zealand has been the development in Spain of a genetically modified myxoma virus that expresses antigens from the coat protein of RHDV. This was developed as a means of simultaneously immunizing wild rabbits against both diseases (Torres *et al* 2000). The myxoma virus used in developing this GMO was selected for its limited ability to spread in natural rabbit populations (Barcena *et al* 2000) but Angulo and Cooke (2002) have highlighted the need for international debate on the use and regulation of such viruses. Obviously, such a live-virus vaccine is a potential risk to rabbit control in Australia especially if it were to become established in the wild. European countries such as Spain, Portugal and France were equally concerned by former research in Australia into the development of GMOs capable of reducing the fertility of rabbits (Robinson *et al* 1997).

Angulo and Barcena (2007) have recently reviewed progress in efforts to gain permission to use the vaccinating virus. The onus is now squarely on Australian bio-security organizations to undertake a thorough risk analysis.

Immune response to high RHDV antigen levels

Trials using transgenic plants suggest that a high concentration of VP60 can to induce immunity in the absence of infection. Predators such as foxes and cats are exposed to massive amounts of RHDV when they eat infected rabbits and they too develop antibodies against RHDV although showing no sign of disease (Leighton *et al* 1995). Experiments with foxes (Gavier-Widen *et al* 1997) and cats fed infected rabbit livers

(Zheng *et al* 2003) could not rule out abortive replication but active RHDV replication was not demonstrated.

Concern has been expressed that RHDV put on baits to initiate new disease outbreaks among rabbits in Australia and New Zealand might immunize rabbits if the virus was killed by exposure to heat or UV-light. However, exposure of rabbits to UV-inactivated virus on baits did not promote antibody formation and immunity (Henning *et al* 2005).

Immune response in vaccinated rabbits

The following Figure shows the antibody responses of laboratory-bred Australian wild rabbits vaccinated on the rump with Cylap HVD. Titres of antibody isotypes resulting from vaccination differ from those resulting from natural challenge. For example, IgA titres are very low in comparison to IgM but rabbits are nevertheless protected from further RHDV challenge.

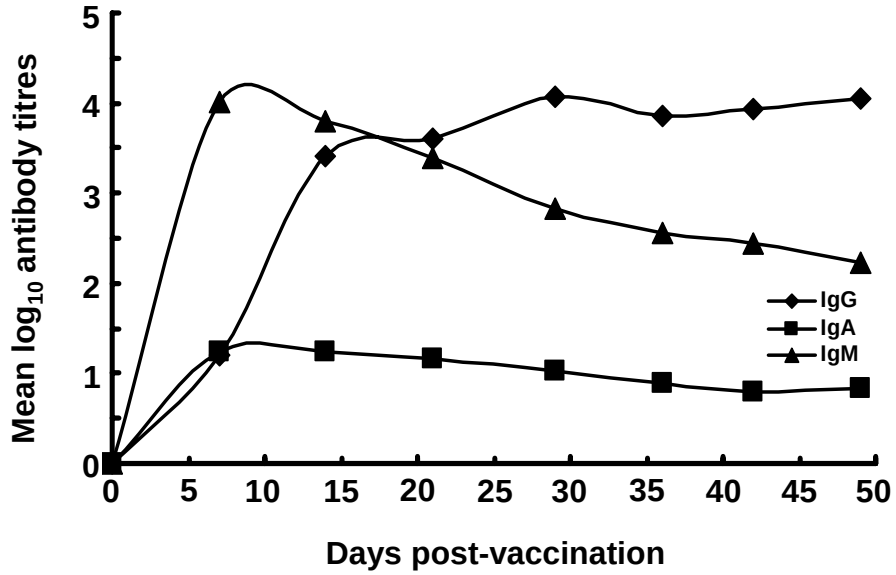


Figure 1. Mean antibody titre (log-transformed) of wild rabbits inoculated with Cylap vaccine. As seen in naturally challenged rabbits, there are relatively brief IgA and IgM responses but IgG antibodies are more persistent.

Re-infection of immune rabbits

Wild rabbits previously exposed to RHD show boosts in IgA and IgG antibody titres, usually in association with new outbreaks of disease among susceptible young rabbits in the population (Cooke *et al* 2000). This almost certainly indicates re-exposure of rabbits to RHDV and it has been shown that vaccinated wild rabbits show strong IgA and IgG responses when dosed orally with RHDV and respond yet again on repeated exposure to the virus (B. Cooke, J. Merchant and C. Musso, unpublished). By

contrast, rabbits re-exposed to the virus by subcutaneous inoculation do not show significant antibody responses.

PCR and Western blotting to detect viral RNA in the blood of the rabbits re-exposed to RHDV show a build up of product in the 48 hours following dosing and this suggests that a low-level, localized infection occurred, probably in the gut mucosa (B. Cooke, J. Merchant and C. Musso, unpublished).

Normally, no disease occurs if immunized rabbits are exposed to virus, but occasionally outbreaks of disease have been seen in poorly immunized rabbits in Europe (Schirrmeier *et al* 1999) and it may be possible that over time antibody titres fall to such low levels that they cannot protect against a heavy dose of virus. However, McPhee *et al* (unpublished) have shown that most adult wild rabbits with detectable antibodies from natural infection show complete protection against a high oral challenge dose of RHDV (1500 LD_{50}).

In other caliciviruses, specifically enteric Noroviruses, human subjects can also be orally reinfected with homologous virus. There is a typical mucosal (IgA) antibody response and repeated infections also boost long-term resistance (Parrino *et al* 1977; Johnson *et al* 1990; Matsui and Greenburg 2000). However, there is a refractory period lasting a few weeks before further infection is possible.

Virus persistence in rabbit tissues

In rabbits that recover from RHD, infective virus is lost from most tissues within 6 days (Pages Mante 1989). Nevertheless, (Gall *et al* 2007) have confirmed using sensitive real-time RT-PCR that viral RNA persists in tissues of rabbits that overcome experimental infection. This explains the widespread observation that viral RNA is recoverable from tissues of rabbits that have previously been exposed to RHD (e.g. Zheng *et al* 2002; Forrester *et al* 2003)

In Australian wild rabbits viral RNA has been readily found in buffy-coat (leukocytes), Peyer's patches and tonsils and is recovered from the liver less reliably Cooke, Lugton and Musso, unpublished). This suggests that some virus particles or at least viral RNA might persist in immuno-privileged tissues and that lymphocytes may take up viral particles that escape into surrounding tissues. It would be useful to determine precisely those immuno-privileged sites where RHDV might persist. Apart from tissues mentioned, the trigeminal ganglia are known to harbour other persistent viruses in rabbits (Rock *et al* 1992). Feline calicivirus (FCV) is persistent in cats but is maintained by persistent infection of the tonsil epithelial mucosa (Dick *et al* 1989).

Viral RNA detected in recovered rabbits has not been associated with the presence of infective viral particles so it is not known whether it might initiate new disease outbreaks. Despite the reported recovery of full length viral genomes from seropositive rabbits (Forrester *et al* 2003), Shien *et al* (2000) found that dexamethasone treatment of experimental rabbits that had recovered from RHD did not lead to reactivation of the virus. This potent immuno-suppressant has previously proved capable of reactivating a wide range of latent viruses including pseudo-rabies virus, infectious bovine rhinotracheitis virus, duck enteritis virus and bovine herpesvirus I.

Virus persistence in the environment

RHDV remains infectious for a significant time in the environment or within dead rabbits. McColl *et al* (2002) found that RHDV persisted in a rabbit carcase for up to 20 days (but not 26 days) at 22ºC. Henning (2003) showed that RHDV on cotton wool was viable at 11 days but not at 44 days whereas RHDV in beef liver (simulating RHDV in a rabbit carcase) was viable for up to three months. Henning's trials using beef liver were done with test samples exposed to natural environmental conditions and daily temperature ranged between 8.8 and 17.6ºC and humidity was between 75 and 92% during the test period. Rodak *et al* (1991) reported that the virus only persisted for 15 minutes at 56ºC and low survival (between 1 minute and 1 hour) at this temperature was confirmed by work at the Australian Animal Health Laboratories (Westbury 1996). The observations of Asgari *et al* (1998) that virus was detectable for up to 9 days (but not 11 days) in the regurgita from blow-flies held at 22 – 25ºC is also consistent with these data

These data are sufficient to estimate that at daily temperatures between 11ºC (minimum) and 24ºC (maximum) when RHDV spreads best (Smyth *et al* 1997) virus in a rabbit carcase would persist on average for about 40 days (range from 128 to 14 days). This seems broadly consistent with the epidemiological modelling of Barlow (1999) who argued on theoretical grounds that RHD would only persist if there is a free-living virus reservoir in the environment in which the viral particles have a half-life of at least 2 weeks.

Henning *et al* (2005) also showed that RHDV in faeces and urine was rapidly made inactive by UV light. Consequently, high UV levels and high soil temperatures in inland Australia during summer would quickly inactivate RHDV on the soil surface. Rogers (1970) showed that in northern South Australia soil surface temperatures could exceed 60ºC for up to 6 hours each day.

Given the inhospitable climate of the soil surface, RHDV is most likely to persist in rabbit carcases within rabbit warrens where, a meter or so underground, diurnal temperature changes are minimal (Cooke 1990). However, burrow temperatures do change slowly during the year, showing a lag of 38 days behind the peaks and troughs of annual solar radiation. At their seasonal peak, burrow temperatures may also be several degrees higher than the reported maximum daily air temperatures because high temperatures reached on the soil surface gradually penetrate deeper layers.

These influences, although appearing small, may nonetheless have important implications. Smyth *et al* (1997) had previously noted that lags in the way the virus responded to climatic conditions at the scale of a population are not understood - but the lagged changes in warren temperatures would offer one explanation for RHD outbreaks persisting into early summer while being slow to build up when equivalent daily temperatures recur in autumn. In a sense, both the field observations and the potential explanatory mechanism can be seen as supporting the idea that carcases of rabbits that died from RHD are a key to understanding virus persistence.

Little is known about the effects of other environmental factors such as humidity (and interactions between temperature and humidity) on virus stability. However, Henzell *et al* (2002) speculate that the low impact of RHD following its arrival in warmer areas of Australia could be explained if combined high humidity and high temperature reduced virus survival.

Genetic variation in RHDV

Nowotny *et al* (1997) sequenced RHDV and EBHSV samples from across Europe and reported 52.6 to 60.0% homology between RHDV and EBHSV but 89.4 to 100% homology between selected viruses within each quasi-species. They considered that this confirmed the two viruses as distinct members of the *Caliciviridae.* Nevertheless, despite the high homology of RHDV they detected three distinct sub-groups within the RHDV samples which they considered compatible with the history of virus introduction and spread.

Le Gall *et al* (1998) independently reviewed changes in the genetic structure of RHDV samples collected over 7 years following its arrival in France in 1988. They also found a limited range of genetic variation and again, samples were clustered into three discernable sub-groups. By 1995 Genogroup 3 viruses had become more common than sub-groups 1 and 2 and a new G3-1 sub-group had emerged.

Their data suggested rapid dissemination of viruses over long distances and they cite the close relationship between a sequence of RHDV first discovered in Britain and those subsequently observed in Bretagne and Normandy. The Corsican isolate from 1995 similarly matched samples collected in the south of France the previous year. In Australia too, RHD spread across the continent in less than 2 years and, apparently un-aided, crossed Bass Strait from Victoria to Tasmania (Fenner and Fantini 1999).

Le Gall *et al* (1998) considered that there had been a relatively slow change in RHDV sequences through time rather than rapid change such as might be expected from immunological pressure on the capsid protein gene. The authors point out that as RHDV is small, large genetic changes are probably unlikely as all components of the virus must co-evolve at a similar rate. Furthermore, the high mortality of rabbits and short duration of disease would not allow for prolonged antibody selection of viable mutants.

Capucci *et al* (1998) described a new consistent antigenic variant that they called RHDVa. This virus had high pathogenicity but did not react with the monoclonal antibody 1H8 commonly used in detecting RHDV. It was suggested that, because most amino acid substitutions in the VP60 occurred between amino acids 344 and 370, the epitope for 1H8 probably lies in that region. Shortly afterwards, Schirrmeier *et al* (1999) showed that RHDV variants from previously vaccinated domestic rabbits often had genetic sequences similar to those of standard reference strains but in a few aberrant viruses amino acid alterations were found clustered between residues 301 and 328 (region C), 344 and 434 (region E) and also in the 3' region of the capsid protein gene. Interestingly, experimental vaccination of rabbits followed by challenge with the heterologous variant strains showed restricted cross-protection against one of the strains.

More recent data, collected from 1999 to 2002 (Le Gall-Recule *et al* 2003), shows that genotype groups 1 and 2 previously seen in Europe have disappeared and three new genogroups (G4 to G6) can now be identified. Genogroup G4 emerged from genogroup G3, which subsequently disappeared and genogroup G5 was a new independent group. The genogroup G6, including RHDVa, contains isolates collected in mainland France, Reunion Island, Germany and USA. Clearly, new variants are replacing older ones on a continental scale. Mutiz *et al* (2006) have confirmed that in eastern Hungary there is also good evidence of several genogroups with RHDVa now replacing earlier variants. RHDVa was identified as the main virus strain in an outbreak of RHD in Cuba in 2004 (Farnos *et al* 2007) and its importance as a new pandemic strain has recently been emphasised by McIntosh *et al* (2007).

In Europe, where there is a large commercial rabbit industry, it is possible that virus evolution is being driven by factors determined by industry practices, including trade and vaccination, as well as natural evolution in wild rabbits. By comparison, changes in RHDV in Australia and New Zealand should be largely determined by its evolution in wild rabbit populations. Forrester *et al* (2004) have shown that RHDV in New Zealand has changed only slowly although virus genomes generally fall in two sub-groups possibly reflecting separate introductions of the virus. It is known that RHDV originating in Australia was introduced into New Zealand by farmers before the Czech strain v351 virus was officially released.

Current studies are also questioning the simple idea that all highly virulent forms of the virus originated in China. Forrester in particular (Forrester *et al* 2006a; Forrester *et al* 2006b) has argued that RHDV has been present in Europe since the 1950s based on the detection of viral RNA in long-preserved rabbit tissue samples. It has been argued that virulent RHDV may have independently emerged on more than one occasion. Nevertheless, Boscuña *et al* (1997) were unable to detect the virus in fixed tissues from Austrian rabbits preserved between 1974 and 1983 although RHDV was present in equivalent samples in 1986 after RHDV was first recognized. Interestingly, their methods also detected European Brown Hare Syndrome Virus (EBHSV) in preserved tissue samples from hares and these pre-dated the earliest recognition of that disease.

Recent results from Australia (J. Kovaliski *et al* unpublished) show that RHDV samples are generally grouped around the initially released Czech-351 strain of the virus but distinct genogroups are emerging. These distinct clusters appear to have evolved progressively over time. The localities from which virus isolates have been collected have mostly been within South Australia. Nevertheless, an outbreak of RHD in August 2006 that covered most of central and western Victoria and eastern South Australia provided a good opportunity for collecting new virus samples. These samples are currently being sequenced to see how RHDV is continuing to evolve. (G. Mutze and J. Kovaliski pers comm.)

An interesting review of this topic has come from Moss *et al* (2002) who sequenced RHDV obtained from stored rabbit sera to show that RHDV has circulated in Europe for at least 50 years, and possibly for centuries or even millennia before the highly pathogenic variant was recognized in China. In the British review, eight variant clusters of RHDV were identified on the basis of serological and sequence data and some viruses, such as Ashington strain, are quite divergent and more closely resemble

RCV than RHDV. Most interestingly, one variant cluster contained viruses found exclusively in Britain, the earliest isolate in this group being from serum stored since 1959, while the most recent isolate was collected in the year 2000. This suggests remarkable virus stability opposed to the steady change reported by LeGall-Recule *et al* (2003) and raises the question as to whether replacement of virus strains in Britain might be occurring more slowly than in continental Europe. However, Moss *et al* (2002) found little correlation between individual virus isolates from particular geographical regions and assumed that RHDV was dispersed very widely by both commercial activities and passive transfer by birds and insects. There is clearly a need to resolve these discrepancies.

Genetic change in RHDV

Mutation and immune selection

The slow changes in the virus genome in Australia over the last decade suggest a relatively stable virus population. In field situations, mutation is countered by strong selective forces and those viruses that conform to a specific pattern are most likely to persist. Additionally, once a virus becomes widely established in an area it is unlikely a new mutant will rapidly displace it unless that mutant has some major advantage.

As an example, Real *et al* (2005) demonstrated that for fox rabies variants in Ontario, Canada, genetic distance from most recent common ancestor (MRCA) was correlated with position along the wave fronts as the disease spread southwards in distinct invasions. They were unable to reject the null hypothesis based on isolation by distance and therefore suggested that changes occurring as the virus spread accounted for 90% of virus variability. This implies that the first virus variants reaching an area become widely established but thereafter, new variants did not establish easily. To some extent this fits with the strong founder effect and slow subsequent rate of genetic change in RHDV seen in Australia and New Zealand.

Nonetheless, Nilsson *et al* (2003) have described how human Norovirus might change rapidly under antibody selection pressure. The case involved a chronically infected patient with severe cellular immunity impairment yet normal immunoglobulin levels. Over the course of a year, sequencing indicated 32 amino acid changes in the virus genome, many of these in the exposed P2 domain subject to immune pressure. Even if most calicivirus infections are normally short-lived and not influenced by immune selection, significant change can occur under some circumstances.

In wild rabbit populations in Australia occasional rabbits have been observed with a prolonged IgM response to RHDV infection, in one case lasting for at least 13 months (G. Mutze, unpublished). The possibility of occasional rabbits being chronically infected with RHDV is therefore raised.

Recombination

Bull *et al* (2005) have shown that Noroviruses may recombine occasionally and this mostly occurs in the region of overlap between ORF1 and ORF2 enabling the ORF1 of one Norovirus strain to be recombined with the ORF2 of another. This could occur

in co-infected cells when the RdRP activity is discontinuous during transcription of a region of secondary structure allowing a switch between virus templates.

As the ORF2 in Noroviruses encodes the virus capsid this could explain how new strains of Norovirus appear and displace those that had previously had high prevalence. Nevertheless, all recombinants so far observed have involved interchanges only within either group I (GI) or group II (GII) Noroviruses (i.e. there are no recorded GI/GII recombinants). Consequently, interchange does not offer an explanation as to why Noroviruses in both groups show the same range of binding patterns to blood group antigens despite being genetically distinct in other regions (Huang *et al* 2005). Possibly, the binding patterns of capsid proteins predate the evolution of distinct Norovirus subgroups.

Related Vesiviruses such as Feline Calicivirus (FCV) also have the main capsid protein genome contained within ORF2 and might therefore be expected to show recombination rates similar to that observed in Noroviruses.

In contrast to the Noroviruses and Vesiviruses, the VP60 capsid protein of Lagoviruses is contained within the large ORF1, consequently it is arguable that there may not be the same chance of recombination due to discontinuity in activity of the RdRP between reading frames and as a consequence we might expect the Lagoviruses to be more conservative. Nevertheless Katayama *et al* (2004) have recently described an apparent recombinant human Sapovirus which, like the Lagoviruses, has the capsid protein genome encoded within ORF1. The recombination occurred at the polymerase-capsid junction so recombination is possible within ORFs as well as at their junctions.

As shown above (*Genetic variation*), genetic change in RHDV has occurred relatively slowly over the last 15 or so years in European wild rabbits without the widespread appearance of obvious new recombinants. Nevertheless, an occasional new and successful recombinant remains a possibility.

Virus variation and pathogenesis: Potential analytical methods

As RHDV cannot as yet be grown in cell culture, it is difficult to ascertain the significance of genetic variation in the virus. However, there have been some interesting developments that could be used in the future to explore variant virus genotypes and their immunological effects and pathogenicity.

Robinson *et al* (2002) successfully used an array of ELISAs to demonstrate that antibodies found in Australian rabbits before the release of RHD were likely to indicate the presence of other RHDV-like viruses. These ELISAs included the use of smooth forms of RHDV as antigens (cELISA-sf) and solid-phase ELISA which effectively revealed immune responses to well conserved epitopes within lagomorph viruses. Similar ELISA arrays were also used (Capucci et al *1996*; Capucci *et al* 1998) to demonstrate immunological differences between RHDV, RHDVa and RCV.

Neill *et al* (2000) were able to develop chimeric feline caliciviruses (FCV) containing capsid domain exchanges and compare their altered neutralizing specificities thereby inferring significant genetic changes to antigenicity. Slimane *et al* (2000) modified the

encapsidation capacity of recombinant RHDV capsules by addition of packaging sequences from the L1 and L2 protein from Human Papillomavirus Type 16 to enable transfection and gene transfer into rabbit RK13 cells. This might provide a means of comparing the properties of genetic variants of RHDV. Most recently Liu *et al* (2006) have directly inoculated in-vitro transcribed RNA modified with a deliberate silent nucleotide change and demonstrated that the same sequence was recovered from the infected rabbit. The transfection of RNA transcripts into RK-13 cells resulted in the synthesis of viral antigens, indicating that the cDNA clones were able to replicate effectively. This stable infectious molecular clone could be useful for developing a better understanding of the molecular biology and pathogenesis of RHDV.

Are rabbits and RHDV coevolving?

Co-evolution model of myxomatosis

In considering the possible development of genetic resistance to RHD among wild rabbits some of the ideas raised in comparable studies on myxomatosis after its release in Australia in 1950 were reviewed.

It is well documented that the myxoma virus attenuated into numerous field strains that caused less acute forms of disease and prolonged the infectious period of sick rabbits enabling more effective spread (Fenner and Fantini 1999). In parallel, rabbits also developed genetic immunity to the disease and today myxomatosis kills about 50% of wild rabbits. Most rabbits still become infected within the first 12 months of life, so resistance does not involve reduced probability of infection; rather it is a strong cellular resistance that prevents disease becoming as generalized as it does in unselected rabbits (Best and Kerr 2000). Wild rabbits with myxomatosis only occasionally show the severe signs that were widely seen when the disease first spread.

Resistance to RHDV infection

The possibility that wild rabbits are developing genetic resistance to RHD is being investigated (P. Elsworth, D. Berman and B. Cooke, unpublished) using the same approach as Marshall and Fenner (1958) to assess developing genetic resistance to myxomatosis. Susceptible rabbits from different parts of south-eastern Australia have been experimentally challenged with standard doses of virus anticipating a lowering of the case mortality rate, longer survival times and lower virus titres in blood or the livers of rabbits that die in comparison to unselected laboratory rabbits.

It was found that wild rabbits from some localities were resistant to oral infection with low doses (1:25 dilution) of Czech-strain 351 RHDV, the strain originally released in Australia. However, there was no evidence that rabbits are better able to recover from infection; case mortality rates remained high, over 90% of rabbits that become infected died, and there was no prolongation of survival times. Rabbits that did not become infected with a low experimental dose of virus were subsequently challenged with larger amounts of virus or by a different route and many died, confirming that they remained fully susceptible and had simply avoided initial infection.

Thus, avoidance of infection rather than dealing with the debilitating effects of RHD appears to offer rabbits the greatest selective advantage. This means that virus-host co-evolution is likely to follow one of two pathways. On the one hand, rabbit resistance may eventually develop to such a point that the virus may no longer be able to circulate effectively and die out or become limited to those parts of the rabbit's distribution in Australia that remain favourable. On the other hand, if RHDV is constantly co-evolving to find its way around host resistance in a biological 'arms race', then it might persist into the future as a useful biological control agent albeit with a reduced efficacy. If RHDV gains its advantage over other lagoviruses by killing rabbits, then it seems unlikely to evolve into a non-pathogenic enteric virus.

Resistance to infection with RHDV appears to be highest in semi-arid parts of Australia, as was the case for the rabbits' development of genetic resistance to myxomatosis (Marshall and Fenner 1960; Williams *et al* 1990; Parer *et al* 1994). Insufficient data have been gathered to give a precise picture, but nevertheless when viewed in conjunction with other information (see *Predictive models*) seem plausible.

Additional work will be needed to demonstrate whether or not the observed resistance to RHDV infection has a genetic basis. This could be done through breeding experiments to show that resistance is heritable, as was done following the introduction of myxomatosis (Sobey 1969). In the meantime, other approaches are being explored, including an investigation of genes considered to be responsible for the binding of RHDV to rabbit tissues as the first step of infection (see *Cell-binding and replication*). Specifically, DNA samples from Australian rabbits used in RHDV challenge experiments are being analysed in France by P. Guillon to look at variation in *Fut2* and *Sec1* genes according to the origin of the rabbits and their apparent resistance to infection following RHDV challenge.

Nevertheless, it is anticipated that the link between virus cell-binding and resistance to infection will not be as clear as seen in the case of some human Noroviruses. Experiments to detect resistance to RHDV infection in rabbits have already shown that resistance mechanisms are not confined to the mucosal surface: rabbits inoculated intra-muscularly, by-passing the mucosa, still showed differences in resistance to infection.

Queney *et al* (2000) found no evidence of a genetic bottleneck (i.e. loss of alleles) in the immediate aftermath of the spread of RHD in a rabbit population in France where about 90% of the rabbits were killed by disease. The lack of major perturbation of gene frequencies associated with the initial spread of RHD means that long-term shifts in the frequencies of specific alleles associated with development of genetic resistance to infection are unlikely to be confounded by reduced genetic variation among survivors. The genetic structure of Australian rabbit populations is also worth considering given that gene flow in arid-zone populations is high and populations are genetically fairly homogeneous while those in wetter coastal areas show less evidence of genetic transfer between social groups (Fuller *et al* 1996).

In asking whether rabbit resistance to infection is out-stripping virus virulence we would expect to see a long-term decline in the proportion of adult rabbits showing antibodies to RHDV. By contrast, if the virus has been able to adapt, most rabbits

might still show antibodies depending on how quickly new strains of RHDV appear. However, a further caveat on this simple picture is raised by events in Europe where a new serological variant of RHDV (called RHDVa) has displaced earlier genogroups of virus on a wide scale (Le Gall-Recule *et al* 2003).

Virulence changes in RHDV

If rabbits are developing resistance to infection as suggested by preliminary work, then it is important to establish whether changes in sequences of field strains of RHDV are indicative of changes that counter resistance in rabbits enabling viruses to maintain their infectivity.

A PhD project by Peter Elsworth (Queensland DPI/University of Canberra) seeks to establish whether present day field strains of RHDV in Australia have greater capacity to infect partially resistant wild rabbits than the Czech strain v351 originally released. If field strains of virus show relatively greater capacity for infection it will imply that they are co-evolving to maintain their relative infectivity.

Despite their conceptual simplicity, experiments of this type are difficult to carry out because the quantity of each virus given to experimental rabbits will need to be closely matched to avoid confusing 'dose' and 'infectivity'. Nevertheless, real-time PCR methods now provide a means of accurately measuring the number of virus copies in a sample and enabling closely matched doses of each virus to be prepared. Other precautions will need to be taken to ensure that the proportion of infectious particles in preparations is uniformly high.

In practical terms, evidence that RHDV is maintaining its relative infectivity would imply that the virus is likely to remain an important biological control agent into the future.

Rabbit biology and population dynamics

Distribution of the rabbit in Australia

Recently, D. Berman (DPI Queensland) has produced a map of Australia showing the broad distribution of rabbits. To help picture the areas infested, the map uses soil polygons defined in the Australian Soils Atlas; those polygons where rabbits have been accurately recorded, e.g. as global positioning system (GPS) co-ordinates, are shown in pink. A further dimension is added to the map by arguing that activities such as release of Spanish rabbit fleas or intensive efforts to record the spread of RHD indicate that rabbits are perceived as a greater problem in some areas that in others. Quantitative data, such as the number of flea releases in a given area, enable ranking of soil polygons and production of a map showing areas where rabbits are most problematic. Although having obvious limitations, *i.e.* a bias towards biological control methods, the map provides a better picture of rabbit distribution and impact in Australia than has previously been available.

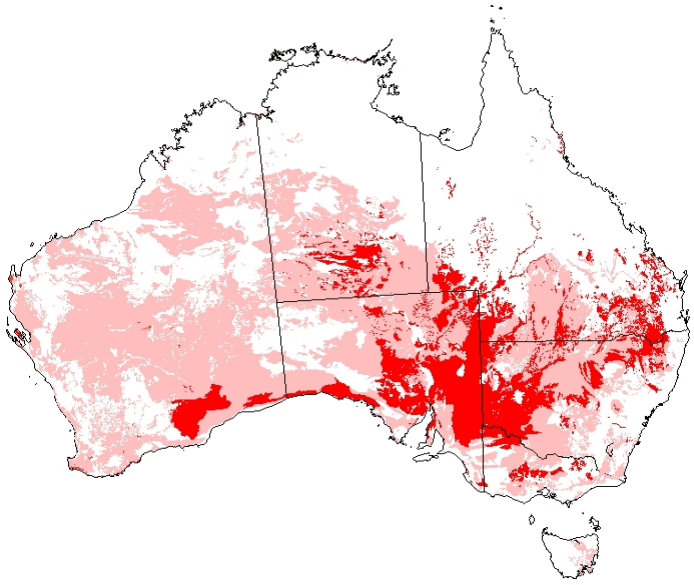


Figure 2. Map showing the broad distribution of rabbits (pink) and regions (red) where major inputs have been made in releasing and monitoring new biological control agents.

The validity and usefulness of the map can be tested against other information relating to rabbit distribution. For example, Cooke (1977) argued that the northerly limits of the rabbit's distribution are likely to be set by reproductive difficulties, especially lactation failure where summer monsoons produce green food needed for reproduction when rabbits are under considerable thermal stress resulting from combined heat and high humidity. Indeed, using appropriate parameters that define the rabbit's physiological tolerances in the climate model CLIMEX (Sutherst and Maywald 1985) it is possible to generate a theoretical distribution that approximates the rabbit's known Australian distribution (Cooke 1992).

Rabbit distribution is further restricted by soil type. Rabbits do not generally do well in areas of heavy cracking clays and poorly drained soils but by contrast favour sandy soils which provide easier burrowing (Parer and Libke 1985; Story *et al* 2004). A combination of climatic and soil factors help define those parts of Australia most suitable for rabbits although other factors such as vegetation quality and growth patterns and land use would enable an ever more detailed understanding.

Rabbit population structure

Rabbit populations vary in structure across Australia. Rabbit populations in arid inland tend to be more continuous because they are not disrupted by intensive land uses such as cultivation of crops. Furthermore, changes in social behaviour and dispersal associated with climatic variability tend to produce genetically homogeneous populations in the inland but stronger genetic structures within social groups in high rainfall coastal areas (Fuller *et al* 1996; Richardson *et al* 2002).

The meta-population structure may provide an indirect indication of the facility with which disease could be transmitted by social contact. For example, in farmland where rabbits are confined to linear habitats such as roadside vegetation or creek-lines surrounded by cultivated land, there is likely to be a lower probability of RHD being spread by rabbit-to-rabbit contact than in an area where rabbit warrens are spaced 50 - 100 m apart over hundreds of hectares and the virus can spread in any direction and re-infest areas as social groups of rabbits make frequent contacts on all sides.

Rabbit behaviour

In Australia, rabbits excavate complex burrow systems or warrens, particularly in arid regions, to provide themselves with protection from extreme temperatures and dryness as well as providing a safe place to escape from predators such as foxes, cats and large raptors. Young rabbits are usually born in nests in the warrens, but when over-crowded a short nest burrow or 'stop' is dug as an alternative. Nevertheless, in New Zealand, rabbits behave differently, usually living in isolated burrows and usually breeding in stops, possibly reflecting the fact that they are subject to a very different suite of predators, namely cats, ferrets and stoats.

Rabbits are also highly social animals and usually form natural groups of 5 – 6 adults (1-2 males, 3-4 females). Such groups occupy a warren or part of a warren and defend a territory that immediately surrounds their core living space. However, when grazing they venture more widely and cannot strictly defend the whole area. Under those circumstances, male rabbits mainly defend females in oestrus that leave the warren to graze. Young rabbits born into the social group normally remain close to their natal warren until forced to leave by declining food resources or the intolerance of older adults as they reach sexual maturity. Territorial behaviour is reduced in summer when breeding ceases and rabbits move about more widely to obtain food among the dry, poor quality pastures.

Members of each social group recognize each other because of their common group odour and males in particular mark females and young with secretions from their chin glands (sub-mandibular glands) or urine. Males also mark territorial areas using faeces coated with strong-smelling anal gland secretions. These are commonly deposited on latrines or dung hills established at various distances from the warren. When visiting dung-hills they sniff recent faeces to detect signs of visitors from other territories and 'chin' to superimpose their scent on foreign faeces. Mykytowycz (1966) considered that marking territories in this way reduced direct territorial conflict.

Female rabbits visit these buck-heaps relatively infrequently and for relatively short periods. They are more likely to urinate on the site than deposit faeces. Young rabbits less than 4 months old rarely visit buck-heaps and when confronted with a buck-heap under experimental conditions show little interest in sniffing or chinning. The odour of dominant adult males further inhibits the young from eating or nibbling of grasses growing from the turf around the buck-heap (Mykytowycz and Hesterman1969).

The number of buck-heaps, their absolute density and the number of faeces on them varies between regions with different climates and can vary seasonally especially

where heavy rainfall and strong pasture growth in spring tend to break down faeces and obscure distinct dung-hills (Mykytowycz and Gambale 1969).

The importance of social and territorial behaviour on the spread of RHD is unclear. On the one hand, if rabbits live mainly within tight social groups the disease may not spread easily, yet on the other hand, some aspects of social behaviour such as sniffing and marking strange faeces could greatly facilitate spread. It is also notable that genetic studies have identified multiple paternities in individual litters of rabbit kittens (e.g. Surridge *et al* 1999) suggesting that territorial behaviour and defence of partners does not exclude the possibility of transmission of RHD through wide sexual contact.

White *et al* (2003) and Moseby *et al* (2005) show that there is great variation in home range, movements and territorial behaviour in Australian rabbits. Up to 30% of rabbits live on the surface rather than in burrows with consequent changes in risk of contracting disease.

Clearly, if social behaviour is important in the epidemiology of RHD, it would be expected that adult male and female rabbits and juvenile rabbits might show different rates of infection and that there may be seasonal changes in epidemiology associated with the onset and cessation of breeding. However, without proper experimental analysis, it would be almost impossible to unravel social behaviour from other factors contributing to disease spread. Climate is a driving force not only of rabbit behaviour but also for virus survival and the seasonal movements and abundance of vectors.

Reproduction

Throughout much of Australia, rabbit breeding is determined by seasonal climatic variables such as rainfall, temperature and day length (Gilbert *et al* 1987). Reproduction begins in autumn or winter soon after effective rains enable the first pasture growth. Pregnancy lasts 28-29 days and immediate post-partum mating often enables females to bear litters at approximately monthly intervals. Breeding usually ceases in late spring (October – November in Australia) as pastures begin to mature and dry off. Each litter usually consists of 4 to 8 young, with bigger litters being produced by larger, older does. Quite clearly, the seasonal production of young results in a cyclical pattern in the abundance of new rabbits susceptible to RHD in the population.

In those parts of Australia with arid and Mediterranean-like climates, rabbit breeding can be accurately predicted by combining rainfall and evaporation data to estimate those periods of the year when soil moisture levels should be sufficient to sustain pasture growth (Cooke 1977, Wood 1980). In cooler, wetter climates the relationship between pasture growth and reproduction is less clear. However, in Britain, rabbits often begin breeding soon after the winter solstice and the first kittens come above ground as spring growth begins. In physiological terms, increasing day length appears to provide a proximal cue to enable rabbits to physiologically ‘anticipate’ improving food quality.

Survival

Food and climate

Young rabbits produced during the winter growing season require good quality green vegetation to maintain high growth rates. However, at the end of the season, pastures mature and become less palatable and less nutritious. Young rabbits that have not built up sufficient reserves starve and disperse and it is mainly sub-adults that survive to become part of the breeding population in the following year.

Wild rabbits do not normally drink but obtain all the water they need from the food they eat. They have moderately powerful kidneys and produce faeces containing only 40% water by weight. They further reduce water loss in the dry summer months by living in relatively cool, humid warrens where respiratory water loss is reduced and there is no need for panting to keep cool. It is only during prolonged drought and overgrazing that moist vegetation may become so sparse that rabbits are unable to maintain themselves. Even then, individual rabbits can lose up to 30% of their body weight before they succumb to the combination of water shortage and starvation.

Other mortality factors

Mortality caused by predation and parasites have seldom been quantified in conjunction with studies of RHD although there are important exceptions e.g. Moriaty *et al* (2000); Reddiex *et al* (2002) and Henning (2005). Nevertheless, in general terms predation of young rabbits by birds, foxes and cats in Australia, and cats, ferrets and stoats in New Zealand is extremely important in limiting rabbit numbers.

Young rabbits in wetter parts of Australia have high infestations of coccidiosis (*Eimeria* spp) (Stodart 1971) and parasitic worms. Nevertheless, Hobbs *et al* (1999) concluded that these parasites were not a major mortality factor in a wetter region of southwestern Australia despite finding some negative relationships between survival and parasite egg and oocyte counts in faeces of individual rabbits.

Epidemiological studies of RHD

Escape from Wardang Island and initial spread

A great deal of information was obtained at the time RHD escaped from Wardang Island. As this has been largely summarized by Fenner and Fantini (1999) it will not be covered again at this stage although some details are discussed in other sections (e.g. *Vector Studies*)

RHD and regional variation in rabbit survival

Neave (1999) collated spotlight-count data from across Australia to assess how RHD had reduced rabbit populations as it first spread across Australia. Despite RHD being a new disease in Australia, it did not affect rabbit survival equally in all areas. An expanded analysis (Henzell *et al* 2002) considered the variables that might explain the observed regional variations in rabbit survival rates.

Two factors were important. First, it was shown that the disease was density dependent, i.e. the proportion of rabbits surviving an outbreak was weakly but inversely related to the population density. Second, it was found that survival showed wide regional variation apparently linked to climatic variables. As many environmental variables are correlated, the analysis was simplified by condensing the large array of potential explanatory variables into fewer principal components.

Survival increased along a gradient from hot, dry areas to cold, wet areas (principal component 1) although there was no interaction with season. This meant that survival was influenced by some persistent factor in each area and was not simply a reflection of the fact that RHD might be more lethal under some weather conditions than others. Henzell *et al* (2002) considered that this could be something such as a pre-existing RHDV-like virus that may have been more prevalent in cold, wet sites and may have partly immunized the rabbit population reducing the effectiveness of RHD. A second possibility is that rabbits are so prolific in cool, wet areas that recruitment of young is sufficient to largely offset the effects of RHD.

A second principal component was associated with an increase in survival from winter dominant rainfall sites to summer dominant rainfall sites although there was a strong seasonal interaction making RHD almost ineffective in summer rainfall areas during summer. After examining a range of possibilities, Henzell *et al* (2002) suggested that this cannot be explained simply in terms of high soil temperatures in summer but might be explained if the virus survived poorly in hot conditions when humidity was also high.

A third component was associated with warmer coastal areas on both sides of the Australian continent but there is no clear explanation and it shows no interaction with season.

In general terms the patterns of rabbit survival observed as RHD first spread across Australia have been maintained in later years. Rabbits remained low in hot dry areas of inland Australia for a decade but in cooler, wetter sites and summer rainfall areas the virus has not had great impact on rabbit populations. This implies that factors evident as RHD first spread continue to influence epidemiology and need to be taken into account in considering present epidemiological studies and predicting future epidemiological trends. In particular, if RHD causes little mortality in some areas, because of competition with putative RHDV-like viruses or other factors that reduce mortality, then it would be expected that rates of development of genetic resistance to RHDV would also be influenced by similar constraints.

Vector studies

The escape of RHDV from quarantine enclosures on Wardang Island was associated with the first few warm days of spring when flies from inland Australia are known to move southward. Results from fly-trapping around the quarantine compound on 19 August and 24 September 1995 showed a 1000-fold increase in numbers of bush flies, *Musca vetustissima*, and substantial increases in blowflies, *Calliphora dubia* and *C. stygia* (McColl *et al* 2005). Some flies taken from traps on 24 September 1995 proved positive to RHDV after PCR analysis and this corresponded with the virus crossing,

some three days earlier, from a pen containing two RHDV-infected rabbits to a 'sentinel' pen of susceptible rabbits.

Although the experiments were closed down at that point and rabbits were rapidly removed from all pens in the quarantine area, RHD was soon observed outside the quarantine enclosure and work began towards eliminating rabbits from the rest of the island. This work was in progress when, on 11 October, a sharp cold-front moved across southern Australia and is thought to have dispersed virus-contaminated flies from the island. Simulated fly movements, based on wind direction and speed during those periods of the day when it was warm enough for flight, were in general agreement with the distribution of subsequent cases of RHD on the mainland (Newsome and Mutze 1995; Wardhaugh and Rochester, 1996; Figure 11.8 in Fenner and Fantini 1999). However, it was in the north-east of South Australia, near the township of Yunta, where rabbits were abundant and widely distributed that the disease first became obvious.

Insects appear to have had an important role in distributing RHDV at that time. Rabbits in the north-east of South Australia have home ranges about 600 m in diameter and, because virus only begins to be shed about 36 hours after rabbits become infected, the maximum rate of contact transmission should be about 400 m/day or about 12 km/month. The apparent rate of virus spread in summer, 9 km/month approximated this rate, but spring-time rates of over 400 km/month (Kovaliski 1998) are well beyond all theoretical expectations. Predators such as cats and foxes that scavenged rabbit carcases also seemed unlikely agents. Although there faeces would no doubt contain large quantities of viable virus (Simón *et al* 1994) the rate of virus spread vastly exceeded their range of normal daily movements. Foxes have core home ranges about 1 – 2 km in diameter and cats likewise (Male and Saunders 2000; Molsher *et al* 2005) so that even if these predators ate infected rabbits on one edge of their home range and deposited faeces on another where they subsequently infected rabbits, a rate of spread of about 500 m/day would barely be possible. This hypothetical rate of spread also makes the unlikely assumption that after allowing for normal passage of foods through these predators the rabbits would quickly make contact with fresh predator faeces, become infected and die within two days. Occasional long distance movements by foxes (Male and Saunders 2000) or cats (Edwards *et al* 2001) are also inadequate to explain the scale and thoroughness of spread of RHDV in the initial phase.

As many millions of rabbits died during the initial outbreak of RHD, their carcases not only provided a source of virus but also an abundant source of food for larvae that developed from eggs the flies laid. The massive 'fly-wave' generated at that time may have assisted in moving the virus about on a wide scale. As spring turned to summer and the virus spread over more of Australia, there were changes in the species of flies that potentially carried the disease. Although bush flies, *M. vetustissima*, were ubiquitous, species such as *C.dubia* and *C. stygia* were replaced by summer-dominant blowfly species such as *Chrysomyia rufifacies* and *Lucilia cuprina* and these became more closely associated with the inland spread of the disease.

Limited PCR studies at the time showed that fly maggots did not retain ingested RHDV on entering pupation so adult flies must become contaminated after alighting on rabbit carcases and eating tissues. However, not all flies are equal in this sense. In

inland South Australia, traps that selectively caught flies entering or leaving rabbit burrows showed that only some species of flies entered rabbit warrens seeking dead rabbits. *Calliphora dubia* and *C. stygia* as well as *Chrysomyia rufifacies* and *Ch. varipes* entered burrows but *Musca vetustissima, Hydrotaea rostrata* and *Lucilia cuprina* apparently did not (J. Hardy, pers com.).

Flies must differ widely in their ability to transmit RHDV. Bushflies, *M. vetustissima*, are known to be capable of transmitting RHDV by feeding on the conjunctiva (McColl *et al* 2002) but do not enter rabbit burrows and are adapted for breeding in cattle dung. They seem unlikely vectors given the rabbits' largely nocturnal behaviour and the fact that rabbits could avoid contact completely by retreating to a burrow. Nevertheless, bushflies may occasionally transmit RHDV simply because of sheer weight of numbers and persistence. By contrast, calliphorid blowflies avidly seek rabbit carcases on which to feed and lay eggs and will enter deep recesses of warrens to find cadavers. They produce faeces and other regurgita containing detectable RHDV for up to 9 days after feeding on RHDV-infected rabbit liver (Asgari *et al* 1998). As a result, if they fed on carcases and deposited faeces on pastures subsequently eaten by rabbits, they could spread RHDV even without contacting live rabbits.

The best experimental studies on vectors come from New Zealand. Barrat *et al* (1998) showed that during RHD outbreaks healthy, seronegative wild rabbits held in wire mesh cages that enabled entry by flying insects became infected with RHDV. They considered that *Hypopygia varia* (Walker) (Sarcophagidae) was the most likely vector and some samples of these flies were found to be positive for RHD. Studies on the seasonal abundance of flies in both the South and North Islands of New Zealand (Barratt *et al* 2001; Henning *et al* 2005) confirm their attraction to dead rabbits and the importance of air temperature in determining activity. In the South Island of New Zealand, fly activity reaches a peak in January (mid-summer) but in warmer and drier environments, such as inland Australia, flies tend to be most active in the spring and autumn and in hot weather they are crepuscular, showing peaks of activity in the early morning and toward dusk. (Norris 1966). This is consistent with analysis of the initial spread of RHD (Smyth *et al* 1997, also shown in Figure 11.13 Fenner and Fantini 1999) suggesting that, generally within Australia, the spread of RHDV would be most rapid in spring and autumn and summer outbreaks would be confined to southern Victoria and Tasmania.

It can be concluded that a number of different fly species potentially spread RHD but their importance is not clear. Bruce *et al* (2004) recorded that RHD spread only sporadically through their study site in WA and no outbreaks were recorded for two years after the disease first spread in 1996 despite the diversity and abundance of flies being similar in all years. From this we can conclude that factors apart from fly abundance must drive disease outbreaks but flies and other vectors could nonetheless help to disperse RHDV when conditions are favourable.

Despite the importance of mosquitoes as vectors of the myxoma virus in Australia, and their ability to transmit RHDV in the laboratory (Lenghaus *et al* 1994) they do not seem to play a major role in transmitting RHD during natural outbreaks in the field. Pools of trapped mosquitoes were shown to be positive for RHDV using PCR as RHDV first spread in Australia and one pool contained viable virus when tested on

susceptible rabbits (McColl *et al* 2005). Nevertheless, as reported in Spain (Villafuerte 1994), the timing of RHD outbreaks in Australia is by no means limited to periods such as late spring when mosquitoes normally increase. Outbreaks of RHD often occur at quite different times to myxomatosis outbreaks which are known to be largely dependent on mosquito vectors.

Indirect evidence that mosquitoes are not major vectors may also be drawn from the antibody responses of immune rabbits re-exposed to RHDV when the virus recurs in populations. The majority of adult rabbits show a clear IgA immune response at such times ruling out the possibility that transmission by biting insects is of major importance. An IgA response only occurs in rabbits re-infected by the oral route, i.e. by rabbit to rabbit contact, ingestion of fly contaminated pasture or possibly flies contacting nasal tissues or the conjunctiva. (see *Re-infection of immune rabbits* above).

Asgari *et al* (1998) showed that single fly spots (crop contents and faeces) from blowflies that had been feeding on RHDV-infected rabbit livers contained enough viral particles (2 – 3 infectious doses) to infect wild rabbits collected near Adelaide. However, recent trials using rabbits from the same area suggest that there may be increases in resistance to infection among the rabbits to the extent that it now requires much larger quantities of viral particles to infect rabbits. The role of flies might decrease if the number of viral particles required for infection exceeded the small quantities contained in fly spots.

Rabbit social behaviour and transmission

Because direct contact between domestic rabbits is a very important means of transmitting RHDV, contact transmission is almost certainly important during disease outbreaks among wild rabbits too. However, wild rabbits are not confined and their social behaviour, especially territoriality, doubtless influences epidemiology.

A territorial boundary may be seen as a barrier to virus spread, especially where contact transmission is important. However, territorial defence involves defecating on dung hills and sniffing the urine and faeces of rabbits from adjacent territories and so should facilitate transmission, with sexually mature males playing an important role. As dominant males rub chin-gland secretions on other group members and spray urine on them as well this should help spread disease within social groups too. For females this risk of infection by visiting dung hills might be lower and kittens would have little chance of becoming directly infected in this way.

There is good evidence that adult rabbits are more prone to RHDV infection than young. When RHD spreads through rabbit populations in the Flinders Ranges, a high proportion of adults immediately show a boost in IgA antibody concentrations, implying oral re-exposure to the virus, before the disease seriously impacts upon young rabbits in the population (Cooke unpublished).

White *et al* (2003) have considered the overlap of areas used by radio-collared rabbits and demonstrated hierarchical contact and transmission structures which could influence rates of spread of the disease. For example, surface living rabbits not established in territories have relatively little contact with other individual rabbits

compared to those living in social groups within warrens. Such ideas are being tested experimentally by measuring contact rates between rabbits (Maija Marsh, University of York, U.K and Agriculture NSW, pers. comm.). Combined with other data on population structure and rates of infection and re-infection of rabbits in different age and social classes, this should provide further insight into the dynamics of the spread of RHD. New technologies used to obtain data include implanted temperature loggers that record pyrexia associated with infection, and proximity collars that record when similarly collared rabbits come within half a meter of each other. The time and identity of interacting rabbits is recorded. Where possible, the cause of mortality is determined on recovery and autopsy of dead rabbits and reading of data loggers. Normal radio-tracking has also been used to determine the home range of collared rabbits and trapping techniques have been designed to obtain the best possible estimates of rabbit population density.

Despite lack of definite evidence favouring either contact transmission or insect transmission, most scientists appear to favour rabbit to rabbit spread as being of greatest importance. Certainly, it is the idea most commonly chosen for epidemiological modelling (e.g. Fa *et al* 2001).

Interactions with RHDV-like viruses

When RHD was first being assessed as a potential biological control agent in Australia, samples of sera from wild-caught rabbits were tested using competition-ELISA to check that they were likely to be susceptible to the disease. Although a high proportion of rabbits succumbed on challenge with the virus, it was nevertheless noted that sera from some rabbits reacted in ELISA tests (Nagesha *et al* 2000) and that rabbits showing this reactivity in ELISA tests were apparently less likely to die following RHD challenge than rabbits without antibodies.

Evidence of this kind is now known to be wide-spread. Cooke *et al* (2000) found that these pre-existing antibodies differed from those typically seen in rabbits that recovered from RHD; they showed very low c-ELISA activity and moderate IgG reactivity whereas in recovered rabbits both c-ELISA and IgG titres were higher and IgA and IgM antibody isotypes were frequently detectable as well. Moreover, the c-ELISA used by Cooke *et al* (2000) differed from that of Nagesha *et al* (2000) in that the monoclonal antibody 1H8 (obtained from L. Capucci) was used rather than a monoclonal antibody developed in Geelong. This fact is important to note because it would also explain why Nagesha *et al* detected these antibodies using a c-ELISA whereas the antibodies detected by Cooke *et al* (2000) characteristically reacted very weakly in c-ELISA yet showed significant IgG titres.

Robinson *et al* (2002) analysed serum samples collected in New South Wales immediately before and after the spread of RHD. They used ELISA tests designed to detect antibodies to well conserved epitopes common to a range of lagoviruses including RHDV, a non-pathogenic rabbit calicivirus (RCV) (Capucci *et al* 1996) and EBHSV and found a high level of reactivity. The weight of serological evidence strongly supports the idea that a related, RHDV-like calicivirus was circulating in south-eastern Australia at the time that RHDV first spread.

Bruce and Twigg (2004) provide good evidence that similar RHDV-like viruses also circulate in south-western Australia, while recently, McPhee, Mutze and Kovaliski (unpublished) have analysed archived serum samples to show that antibodies to a putative RHDV-like virus were present in rabbits in Victoria in the 1970s and in South Australia and Northern Territory before RHDV was introduced in the 1990s.

Both the archived samples and more recent epidemiological studies show that these characteristic antibodies are very common in rabbit populations in temperate Australia but in drier areas they appear seasonally, or at irregular intervals. Nevertheless, when they do appear they are seen is a significant proportion of young rabbits over several consecutive months. This might be expected if a virus briefly spread through rabbit populations under favourable conditions before dying out once more.

Case studies on individual rabbits live-captured repeatedly over several months also suggest that rabbits often become infected with this putative RHDV-like virus well before they are challenged with RHD. For example, one young rabbit lost its maternal antibody protection then showed antibodies to the putative RHDV-like virus before being found freshly dead and confirmed as having been infected with RHDV.

McPhee, Yoon and Butler (unpublished) have recently reviewed data from challenge studies carried out on wild-caught rabbits of different antibody status that included rabbits judged to have antibodies to a putative RHDV-like virus. Although these antibodies confer some protection against RHDV they only protect against large challenge doses (1500 LD_{50}) if present at relatively high titres.

Cooke *et al* (2002) further argued that pre-existing antibodies appear to be more common in cooler, wetter parts of Australia and may in fact help explain why RHD did not have the heavy impact on rabbits in those areas in comparison to dry inland regions. Henzell *et al* (2002) also thought that one of the principal components in their explanatory model may reflect levels of antibodies to the RHDV-like virus. They found that survival followed climatic trends (lower in hot, dry areas and higher in cooler, wetter areas) but there was no seasonal interaction as might be expected if weather factors such as temperature or rainfall directly influenced disease outcomes. On this basis, it is likely to be a more persistent factor, such as the presence of an interacting virus, whose presence is correlated with general climate rather than the weather itself.

Despite compelling circumstantial evidence based on antibody data, it is only recently that Tanja Strive (CSIRO Entomology) has isolated a new lagovirus from rabbits at Michelago in the Canberra region. From limited sequencing so far this appears to have a 77% similarity with RHDV but greater affinity with RCV. The next step is to describe it and determine whether inoculation of susceptible rabbits causes infection and production of antibodies similar to those assumed to indicate infection in wild rabbits.

It will be important to test the protective nature of antibodies to the RHDV-like virus using low oral doses of RHDV to confirm whether they can protect against infection sufficiently to interact with RHDV in an epidemiological sense, confining outbreaks to part of the year.

Interaction with myxomatosis

Mutze *et al* (2002, unpublished) have shown that with the advent of RHD, myxomatosis now tends to occur less frequently or later in the year (autumn) than was previously the case and this may in turn change its effectiveness.

In the Mount Lofty Ranges, South Australia, the occurrence of myxomatosis had been significantly reduced since RHDV arrived and a high proportion of adult rabbits have no antibodies to myxoma virus (MV). Rabbits are becoming more abundant rather than being reduced apparently because mortality caused by RHD has been insufficient to off-set the reduced mortality from MV. This type of interaction may be limited to specific areas but nevertheless is worth further investigation.

RHD and predators

RHD should be seen as one more factor which helps to control rabbits adding to the previously known factors such as predation, food quality or diseases such as myxomatosis or coccidiosis.

Recent studies re-emphasize the need to maintain RHD in its proper perspective. Moriaty *et al* (2000) showed that in central western New South Wales RHD accounted for 16% of the adult rabbits they followed with radio transmitters whereas predation accounted for 44% and 2% died from other diseases (peritonitis). Foxes killed 28% of rabbits, raptorial birds killed 10% and cats accounted for 6%. For 9% of rabbits the cause of death could not be ascertained.

Reddiex *et al* (2002) measured mortality among radio-collared rabbits both in areas where predators (cats, stoats and ferrets) were controlled and in areas where no predator control was undertaken. RHD broke out during the study enabling interactions between predation and disease to be discerned. Rabbit populations declined or remained steady throughout the study instead of increasing during the breeding season as expected but 18% of radio-collared rabbits survived on sites where predators were reduced whereas none survived on sites where predators remained at normal levels. Predation of nestling rabbits was also significantly higher in those areas where predators remained abundant. Henning (2003) also concluded that RHD was not as important as predation, mainly by cats, as a mortality factor in North Island, New Zealand.

Sandell (unpublished) has recently shown that in north-western Victoria, the implementation of widespread fox control coincided with a significant rise in rabbit numbers. However, on an adjacent area where foxes are not poisoned, rabbits are also building up albeit a little more slowly. Sandell's data suggest that in north-western Victoria RHD may no longer cause sufficient mortality to keep rabbits in check and rabbits will increase even more rapidly if other mortality factors are also withdrawn. Clearly a combination of predation and disease is important for holding rabbits down at very low levels.

Epidemiological field studies

Europe

In Europe, the initial spread of RHD into wild rabbits was linked so closely with the spread in domestic rabbits that there is little possibility of unravelling the key factors involved to obtain insights into epidemiology. Disposal of waste from rabbitries and the use of fresh cut herbage to feed domestic rabbits no doubt provided routes for spreading RHDV in both directions between wild and captive rabbits. Nevertheless, observations in domestic rabbits clearly established the importance of transmission through direct contact and fomites and seasonal patterns of epidemics were seen indicating that climatic variables might be important. The possibility of insect transmission was also explored and flies of the genus *Phormia* were found to transmit the virus with very few viral particles needed to infect a rabbit via the conjunctiva (Gehrman and Kretzschmar 1991). There was also considerable conjecture about seabirds being involved in RHDV transmission in northern Europe particularly when RHD appeared on off-shore islands

Once RHD became established in wild rabbit populations in Europe, a number of studies recorded the initial impact.

Spain

The earliest outbreak of RHDV among wild rabbits was recorded in the south-east of the province of Almería in arid southeastern Spain in June 1988 (Cooke 2002). Subsequently, the disease spread into the province of Murcia, to the north of Almería, by December 1988. Professor Leon-Vizcaino (pers comm) began field studies taking blood samples from live-captured rabbits every 3 months. He showed that many of the surviving rabbits, both adult and sub-adult, carried antibodies detectable by haemagglutination inhibition (HI) tests. During the following year, it was confirmed that some very young rabbits carried antibodies against RHD, but these were considered to be temporary maternal antibodies because all sub-adults were seronegative. The proportion of rabbits carrying antibodies declined as more and more young entered the adult population. By January 1990 no seropositive rabbits were caught but in May 1990 seropositive rabbits were again observed indicating a second disease outbreak had occurred on Vizcaíno's study site.

The spread of RHD across Murcia was patchy but a broad front could be recognised with the disease spreading at about 15 km/month. Advantage was taken of this situation to compare an RHD-affected rabbit population near Bullas with another RHD-free population nearby. Using transect counts to follow changes in rabbit numbers and detect cadavers, it was shown that from mid-June to mid-July 1990 RHD reduced rabbit numbers by almost 50% in comparison with the uninfected site. Haemagglutination (HA) tests were used to confirm RHD in cadavers found in the affected area and haemagglutination inhibition (HI) tests were used to detect antibodies in those rabbits that survived. Neither dead rabbits nor rabbits with antibodies were detected on the control site.

At about the same time, the spread of RHD was followed even further north through Alicante, a third adjoining coastal province (Peiró and Seva 1991). Again RHD spread at about 15 km per month from an outbreak in the south that began in late October 1988 (autumn) and eventually merged with a second outbreak towards the north of the province. Not all rabbit populations were affected as the disease spread; some hunting reserves remained untouched and there was a sharp decline in disease activity at the start of summer. By counting rabbits along standardised transects it was shown that on one site the peak counts in June each year fell from 21.1 rabbits/km in 1988 to 5.2 rabbits/km in 1989 then recovered to 21.2 rabbits/km in 1990. Following the initial outbreaks there were less intensive, localised outbreaks of RHD in the late winter (February-March) of 1990 and the spring (April-May) of 1991(V. Peiró, pers comm) but within individual populations it is possible that outbreaks occurred every second year.

Despite its relatively rapid march through Almeria, Murcia and Alicante, RHD took over 5 years to reach most wild rabbit populations across the Iberian Peninsula. Even though RHD was first reported from Portugal in 1989, the initial spread of RHD through Doñana National Park in south-western Spain only occurred in March-May 1990. Radio-collars were fitted to rabbits to follow its spread in Doñana and it was recorded that 55% of adult rabbits died with both sexes being equally affected. It was also considered that high temperature in the area in late spring and summer may have curtailed the epizootic. However, RHD epizootic at Doñana did not appear to be associated with seasonally high mosquito numbers and it was concluded that vectors did not play a decisive role in transmission.

A broad assessment of the final impact of RHD throughout Spain (Blanco and Villafuerte 1994) showed that although some rabbit populations were able to recover despite added mortality others were heavily suppressed and in general rabbits were held well below normal levels.

Initial observations in the maritime provinces of eastern Spain suggested a very patchy spread of disease and erratic recurrence of the disease every two years or so in most populations. By contrast, Calvete *et al* (2002) studied rabbits in an arid environment in the Ebro River valley near Zaragosa, north-eastern Spain. Regular annual outbreaks of RHD were associated with the winter breeding period. Eighty to ninety percent of adult rabbits had antibodies against RHD although 22% of radio-collared adult rabbits died from RHD. Clearly, not all rabbits were infected while young, but the lack of consistency between the presence of apparent antibodies to RHD and death from the disease raises questions about the reliability of the ELISAs used or suggests that cross-reactive antibodies detected in the rabbits may not have provided full protection against RHD. In short, it raises the possibility that RHDV-like viruses might also circulate in the population although Calvete considers there is no general evidence for this on his study sites (see *Origin of RHD*).

Experimental manipulation by vaccinating captured wild rabbits against RHD (Calvete *et al* 2004) showed that unvaccinated young were 13.6 times more likely to die than vaccinated rabbits. This suggests that mortality from RHD in infected rabbits must have been about 93%. Rabbits which had no natural antibodies to RHD were 5.2 times more likely to die than rabbits with unequivocal antibody titres, suggesting that RHD probably accounted for over 80% of them. In adults, vaccination did not

significantly reduce mortality, presumably because the majority were already naturally immune.

Recent data from Calvete *et al* (2006) show that rabbits in Teruel Province, to the south of Zaragoza, generally remain low. Nevertheless, there is some evidence of a substantial relative increase in those populations that were hit hardest by the virus in the first instance. This may be consistent with observations in Australia where rabbits now appear to be increasing in those areas where RHD initially had its greatest impact (see *Predictive models*).

France

In France where the disease was first noted among wild rabbits in 1989, recurrent outbreaks of RHD were observed among wild rabbits in the Carmargue, Vaucluse and Héralt in the south. The incidence of the disease was carefully monitored on a national scale but it was not until 1995 that the first outbreak of RHD was seen at the Chèvreloup arboretum, near Paris, among rabbits monitored since 1989 (Marchandeau *et al* 1998). The Chèvreloup rabbit population declined to 12% of its initial level in the course of a year and has been slow to show signs of recovery.

Analysis of blood samples from a population at Cerizay in Western France revealed that many rabbits that died from RHDV not only had detectable virus but also antibodies detectable in RHDV ELISAs. As these antibodies could not have been formed as a result of the brief acute infection, it has been argued that the rabbits must have carried antibodies to an RHDV-like virus although these did not confer enough protection to prevent the rabbits from developing acute RHD (Marchandeau *et al* 2005). Since then, an RHDV-like virus has reportedly been isolated from this region in France (J. Le Pendu pers comm.)

Britain

RHD was first recorded in domestic rabbits in Britain in 1992 and outbreaks in wild rabbits were recorded from August 1994 onwards. Trout (1999) collated information on the locations of confirmed outbreaks in wild rabbits over the following 2 – 3 years and showed that most occurred between June and December. He followed RHD epidemiology in three populations noting that numbers fell in two populations. On the 130 ha island of Ramsay off the coast of Wales, serum samples taken from rabbits in the winter of 1994 showed that only 18% or rabbits had protective antibodies against RHDV. Subsequently, an outbreak of RHD that began in July 1995 killed some 5000 rabbits over the following 3 weeks (i.e. about 40/ha). Nevertheless, in most wild populations sampled, rabbits had antibodies to pre-existing RHDV-like viruses that were protective against acute RHDV infection and this was thought to lower the impact of disease (Chasey *et al* 1997).

Additional epidemiological information on wild rabbits in Britain comes from White *et al* (2004) who presented evidence of RHDV transmission during and just after the breeding season and suggested that most rabbits are infected before they reach 10 weeks of age. The idea of early infection with the virus fits with the model suggested by Calvete (2006) to explain why RHD has little impact in some rabbit populations

although there was uncertainty that the antibodies shown in individual rabbits had resulted from challenge with virulent RHDV or other attenuated strains.

White *et al* (2004) also compared the genetic sequences of virus samples obtained from rabbits that died from RHD with those of rabbits that had survived challenge yet still harbored circulating viral RNA. They argued that there may be both virulent and avirulent 'modes of behaviour' of the same viruses because they obtained very similar viral RNA sequences from rabbits that died from acute RHD or those that had sero-converted and remained healthy but retained traces of viral RNA.

Nonetheless, it seems unnecessary to argue along these lines. This would also be the case where any strain of RHDV killed only a portion of the population. Where variations in viral dose, the rabbit's age at infection and the host's immune response can modify the outcome following infection, there seems little reason to invoke specific molecular machinery within the virus.

The initial impact of RHD on wild rabbit populations in Europe seems to have been strongly influenced by geography and climate. Certainly, the greatest recorded declines in rabbit abundance have been in Spain, Portugal and France whereas the virus did not so severely reduce rabbit populations in Britain or other countries of northern Europe, possible exceptions being major declines of rabbits reported from the coastal dunes of Holland (Bijlsma 2004).

Australia and New Zealand

New South Wales

Saunders *et al* (1999) describe patchy results as RHDV spread through central western NSW. The rabbit populations at Burrendong and Euchareena fell by 91% and 68% respectively as RHD arrived but near Bathurst, the population actually doubled, possibly due to RHD arriving later when rabbits had already begun breeding. This population has since remained high.

Richardson *et al* (2007) showed that at Cattai, western Sydney, RHD failed to establish naturally and repeated deliberate releases also failed. The most likely explanation was considered to be the presence of antibodies to an RHDV-like virus in most adult rabbits. These antibodies were prevalent in rabbits throughout the study, even in blood samples collected in 1992, well before the spread of RHD in Australia. The studies in the Bathurst and Sydney areas established clearly that from the outset the capacity of RHD to reduce rabbits in eastern Australia was limited.

Studies near Bathurst (Moriaty *et al* 2000) using radio-collared rabbits nevertheless showed that 16% of adult rabbits died from RHD and that most of these deaths occurred in July, about the time that breeding would have commenced. This pattern suggests that not all rabbits are affected with RHD while very young.

It is interesting that the Spanish study in the arid Ebro valley about 22% of radio-collared adult rabbits were recorded as dying from RHD. Expressed in terms of percentage mortality the two data sets seem to be rather similar, however the Bathurst

population was able to maintain itself around its initial level whereas the Ebro valley population had generally declined. Nevertheless, if the rabbit population near Bathurst was more productive that that in the Ebro Valley, sufficient numbers of young might still survive to maintain a high breeding population.

Queensland

Storey *et al* (2004) reviewed the spread of RHD across Queensland between October 1995 and October 1996 and monitored six populations of rabbits for at least 2 years to establish trends in the populations. The initial impact of the disease was highly variable with some populations declining sharply and others increasing. However, within 30 months, spotlight counts showed that rabbits had generally declined by 90%. After adjusting for seasonal conditions using a population simulation model, it was estimated that rabbit density in general had declined by about 74% (range 43 – 93% depending on the sites).

No outbreaks were observed when the density of susceptible rabbits was less than 12 km^{-2} and the authors concluded that RHDV may not persist if the density remains low for long periods. It is worth noting that the critical density of susceptible rabbits was calculated from spotlight counts and therefore may under-estimate the true population density. Cooke (unpublished) found that during spotlight counts only about one-third of the estimated rabbit population was seen on a study site at Witchitie Station, South Australia. Presumably not all rabbits are above ground at any given time and many may hide when an approaching vehicle and light are detected. If this also applies to the Queensland data it is possible that RHD does not spread if the density of susceptible rabbits falls below about 36 km^{-2}, or 0.36 ha^{-1}. Nevertheless, this represents one of the few studies for which a threshold density for RHD spread has been suggested.

South Australia

Kovaliski (1998) described the initial spread of RHD through South Australia and adjoining states and Mutze *et al* (1998) described the initial outbreak in semi-arid north-eastern South Australia in which over 90% of rabbits died.

As found in semi-arid parts of Spain, Cooke *et al* (2000) showed that in inland South Australia outbreaks of RHD occurred each year, often in autumn and winter after initiation of breeding, but also among young susceptible rabbits in spring. Nevertheless, many late-born young enter the summer period without becoming infected and subsequently are challenged with the virus at the start of the following breeding season when high mortality results.

From individual case histories it is clear that some young rabbits are challenged with RHD while they retain maternal antibodies while others are clearly challenged after maternal antibodies have declined to undetectable levels. Nevertheless, the majority of young rabbits simply disappear from the population without providing any clear information on whether their serological status at the time of infection improved their chances of survival. The best chance of understanding the importance of early infection or maternal antibodies lies in using population data and detailed analysis of different cohorts of rabbits during the breeding season to look at survival trends.

Data from shot samples collected at Manunda in South Australia strongly suggest that RHD outbreaks are linked to the presence of susceptible young adult rabbits in the population (Cooke unpublished). The presence of susceptible juveniles alone does not appear to be adequate to sustain an epizootic. This might be linked to the restricted movement of young rabbits within their natal territory but could also be linked to differences in the development of disease. Shien *et al* (2000) for example found that infected young rabbits began shedding virus in urine and faeces about 12 hours later than infected adults and this might mean that the rate of spread of virus among young rabbits is lower than for adults.

A 12-year study of RHD at Turretfield in the mid-north crop-lands of South Australia R. Sinclair (unpublished) has provided evidence of a two year cycle of disease outbreaks or irregular outbreaks similar to those described in south-eastern Spain and implied for Western Australian crop-lands (Bruce *et al* 2004). Virus appears each year but only builds sufficient momentum to become epizootic in some years.

The reasons underlying the sporadic occurrence of disease remain unknown but could reflect the fragmentation and structure of rabbit populations in farmlands (confined to 'linear' habitats such as creek-lines, road and vegetation reserves) which means that virus frequently dies out and only arrives by rabbit-to-rabbit contact relatively infrequently along restricted routes. It may not necessarily spread well unless conditions are ideal.

Mutze *et al* (2002) developed a generalized conceptual model of how RHD affects rabbit populations in southern Australia based on comparisons of rabbit population structures and breeding patterns before and after the spread of RHD. Reproductive patterns remained constant, driven largely by climate, but disease activity that commences a month or two after breeding begins and continues into the winter and spring severely suppresses populations. Nevertheless there is compensatory recruitment of young born late in the breeding season and rabbit abundance increases in the early summer. There is a corresponding shift in the age-structure of the population associated with these epidemiological changes and the delayed recruitment of young sub-adult rabbits into the population also means that the seasonal peak in myxomatosis activity is pushed back from spring into early summer.

Victoria

S. McPhee (unpublished) carried out a five year epidemiological study of RHD in a dense rabbit population at Bacchus Marsh, Victoria. Initial results suggest that, although active during the rabbits' breeding season, RHD appears to cause little additional mortality of young and it is not until the breeding season ends in late spring and early summer that RHD has any measurable effect on survival (Butler, Yoon and McPhee unpubl). However, by that time all early born young have acquired antibodies to RHD and generally survive the summer very well as young adults. They are the main group recruited into the breeding population each year. Recruitment of young adults appears to be adequate to maintain the population at a level not much lower than pre-RHD densities.

Western Australia

The best analysed field studies so far have been published by Bruce *et al* (2004) and Bruce and Twigg (2005). RHD was monitored across 9 sites after it began to spread into the south-west of Western Australia in early 1996. It had its greatest impact in arid and semi-arid areas bringing rabbits down to about 10% of their former numbers but in higher rainfall areas results were patchy and the disease often spread without much obvious impact. At Gingin for example, it took 3 years before the virus was recorded from all three study areas despite the fact that they were less than 8 km apart.

At Kojaneerup, a high rainfall area near Albany, the initial outbreak in 1996 was an isolated incident and no further clear outbreaks occurred during the following three years although some seropositive rabbits were detected. Some limited spread of the virus was noted in 1999 and again in 2001. Importantly, an outbreak of RHD at Kojaneerup was apparently initiated in 1999 by releasing rabbits deliberately inoculated with RHDV (Bruce and Twigg 2005). Such data would suggest that the virus does not persist within local populations and the natural reintroduction of the virus into this area is extremely limited despite rabbits being susceptible. However, not all rabbits recovered from the out-break zone were infected by the same virus isolate, indicating that natural spread of RHD may occur at low levels and be difficult to detect .

Taking such evidence together with data from other sites such as Turretfield in South Australia (see above), where outbreaks also occur sporadically despite apparent susceptibility of rabbits, it is arguable that RHD outbreaks in some areas may be limited by the capacity of the virus to persist and spread effectively. A range of possibilities might be invoked to explain this, including the direct effects of some climatic variable such as relative humidity on viral persistence or indirect factors such as the presence of another RHDV-like virus which reduces the chance that rabbits will develop acute RHDV and the likelihood that they will shed large quantities of virus to infect other rabbits. However, the demonstration that an outbreak could be initiated by inoculating rabbits favours the idea that RHDV does not persist in local populations at Kojaneerup and re-establishes itself in them relatively infrequently.

New Zealand

Parkes *et al* (2002) considered that in New Zealand's South Island RHD has become widely established and occurs in either annual or biennial epidemics that begin in spring, affecting susceptible adults, then persisting through to autumn affecting mainly young born during the previous breeding season. Rabbit populations were reduced by about 90% in some areas where outbreaks occurred regularly and held at about 50% of former levels where disease occurred less frequently or affected a lower proportion of the population.

Henning (2003) in a detailed study over 3 years showed that in the North Island near Palmerston, RHD appeared each year but only caused high mortality in the first and third years. RHD outbreaks clearly show some of the same characteristics and patterns as those noted in other parts of the world (e.g. biennial outbreaks noted at

Turretfield, South Australia and in south-eastern Spain) and the spread of virus through young adult rabbits early in the breeding season.

Reddiex *et al* (2002) showed that the timing of outbreaks of RHD can be critical in influencing the outcomes of epizootics. Using radio-collared rabbits, they showed that that early born young of the breeding season survive well, however, such animals were fully susceptible if RHD spread naturally in late October and early December and consequently suffered high mortality.

Epidemiological models

Mathematical models

An analytical model for RHD epidemiology

Despite a wide range of epidemiological studies of RHD in wild rabbits, there are relatively few live-capture studies that allow for repeated sampling of individual rabbits to provide direct insight into disease behaviour based on rabbit survival and serology. But even then, the enormous amount of data collected and the handling of data in a statistically acceptable way requires the development of new approaches for analyses.

Program MARK estimates survival of rabbits in a population based on captures and recaptures of individually marked (ear tagged) rabbits using the Cormack-Jolly-Seber model. From the capture history of the rabbits a set of capture (*p*) and survival (*phi*) probabilities are estimated prior to detailed analysis. Explanatory factors contributing to survival (e.g. body weight (age), sex, time of year, immune status) are used to develop a model using logit link on standardized data. The model allows the inclusion of covariates and interaction terms. The best predictive model is developed by adding or rejecting terms on the basis of change of deviance tests with a χ^2 distribution approximation.

So far, data from two study sites, Bacchus Marsh in cool, temperate Victoria and Gum Creek in semi-arid South Australia have been analysed (K. Butler, H.-J. Yoon and S. McPhee pers. comm.). Outputs from these models enable calculation of seasonal fluctuations in the numbers of young rabbits in different age classes and subsequent changes in the size of the adult population. It is also possible to construct life tables using survival rates of rabbits in different age-groups and serological classes to understand how young rabbits are recruited into the breeding population despite the presence of a highly lethal disease.

Comparison of the models for the two sites, although incomplete, has already highlighted some major differences, including the strong seasonal pattern of RHD at Bacchus Marsh and the less seasonal and severe impact of RHDV at Gum Creek. Analysis confirmed that RHD was a major factor among others (e.g. predation and myxomatosis) that help regulate rabbit populations. At neither site was its impact trivial.

The model for Bacchus Marsh shows that survival can be explained in terms of capture history, body weight (age), cELISA titre, the presence of clinical myxomatosis, and IgG titres. However, terms rejected from the model are also revealing as they often confirm expectations from other studies. For example, the absence of gender as an important variable might be expected given that there is little published evidence of differential infection or mortality between rabbits. The absence of IgM antibody titres among the significant explanatory variables is also understandable given their transitory nature in rabbits that recover from primary infection. They are less reliable than cELISA titres in indicating immunity from acute disease.

The Bacchus Marsh analysis confirmed that RHD was a major cause of mortality in young rabbits during the summer months, a fact not apparent from other analyses such as the presence of IgM antibodies among captured rabbits. Quite clearly, if most susceptible rabbits die as a result of infection there would be few survivors showing IgM antibodies. On the other hand, even a relatively low level of infection among young rabbits fully protected by maternal antibodies might result in significant numbers of them with obvious IgM titres.

Analytical models of this kind are limited by the data available and often raise questions that may be unanswerable without seeking further information. For example, little can be concluded about disease activity and survival of young rabbits newly emerged from the maternal nest because few rabbits in that age group are captured and survival is low. The spread of RHD among this age-class may be important from an epidemiological point of view but nonetheless remains difficult to analyse without more detailed intensive studies.

Ultimately, analytical models should provide realistic basic data needed for epidemiological simulation models. These include accurate population estimates, natural survival rates of different age classes and estimates of infection rates and mortality rates.

Experience with analytical modelling suggests it is a very useful tool for deriving accurate information by controlling error estimates but it represents only one part of the complex task of gaining insights into epidemiology.

In considering how to progress beyond analytical models it is important to visualize not only how RHD takes its toll but also consider how some rabbit populations maintain relatively high numbers despite being afflicted by a highly lethal disease.

General epidemiological models

There have been a number of attempts to model the epidemiology of RHD to show its broad features and evaluate influential factors. These are normally derived from the (Susceptible-infected-resistant or SIR) disease-host model (Anderson and May 1979) and parameters such as virus decay rate, transmission coefficients and disease mortality rate are set where possible from published literature. In the case of rabbits it is important to include seasonal patterns of breeding in the models to mimic the appearance of new susceptible rabbits in the population.

Barlow (1995) and Barlow and Kean (1998) derived a model to show how RHD might affect Australasian rabbit populations and subsequently showed (Barlow *et al* 2002) that it gave reasonable agreement with field data in terms of gross population changes following the release of RHD in New Zealand. Nevertheless there were deviations from the model's predictions including inconsistencies between mortality rates and the level of immunity in populations in the modelled population compared with what happened in the field. Natural disease outbreaks also occurred more irregularly and later than expected if the main driving factor was the abundance of susceptible young.

Possibly of more importance was the observation that the inclusion of juvenile resilience in the model was important because without it the time between outbreaks was longer than observed (i.e. every second year instead of every year), however adding maternal antibody protection to the model had little additional effect.

Fa *et al* (2001) developed an individual-based epidemiological model more detailed than that of Barlow and Kean (1998) that provides for movement of rabbits between local populations and territories. In broad terms it gives an approximation to the kinds of changes seen in rabbit populations following the introduction of virulent RHD but these authors conclude that it is difficult to assess their model in the absence of any detailed field data on epidemiology. Presumably, these kinds of models might be better refined as data from long-term field studies become more widely available.

One clear advantage of these complex individual based models is the ability to build in behaviour such as dispersal of young and provide for different dispersal rates between male and female rabbits. This may prove to be an important attribute if transmission of RHD is shown to be facilitated by some age-classes of rabbits more than others.

Alternatives to host based models might provide a better basis for understanding the epidemiology of RHD which is clearly influenced by climatic constraints as much as host density. Milner-Gulland *et al* 2004 developed a model for *Echinococcus multilocularis* which used a parasite-focused approach rather than the usual host-based approach. This enables modelling of the effects of climate on parasite survival (eggs in fox faeces) as well as host density in patchy habitats. The most favourable habitat occurs where climate allows prolonged survival of eggs (low temperatures and high humidity) with a high density of hosts (gerbils/ha) that may become infected.

Calvete (2006) developed a model based on his detailed observations and epidemiological studies in Spain in which he sought to explain why some rabbit populations in Spain are being held low by RHD yet other populations have shown little disease impact and retain high populations. His model is based on the idea that the infection rates are high in dense rabbit populations with the consequence that rabbits are generally infected when young and so have higher survival rates due to age-related resilience. In low density populations rabbits are older when infected and consequently suffer high mortality. Other factors such as carrying capacity, that sets population density, and the productivity of the populations would importantly influence disease impact according to the outcomes of his model.

Current research in Australia, particularly the analysis of the Bacchus Marsh field study, suggests a different basis for the maintenance of high populations to that proposed by Calvete (2006). RHD spreads most rapidly at Bacchus Marsh during summer, not during the rabbit's breeding season when disease spread appears to be quite low. Nevertheless, enough young rabbits contract RHD while protected by age-related resilience and maternal or RHDV-like antibodies to become a significant fraction of the immune breeding population. In short, the concept of young contracting RHD during their early life still remains an important recruiting mechanism but the timing of disease spread is driven by factors other than rabbit population density. The high productivity of the Bacchus Marsh population probably enables effective recruitment despite heavy losses to disease.

While there is considerable evidence that the timing and impact of outbreaks of RHD are dictated by climatic factors (Smyth *et al* 1997) it is more difficult to clearly establish that rabbit density is an important driving force in epidemiology. Nevertheless, Henzell *et al* (2002) showed that rabbit survival rates were partly explained by population density when RHDV first spread. Mutze and McPhee (unpublished) have also shown that in experimental populations where rabbits had been controlled by poisoning and warren ripping, the proportion of young rabbits with antibodies to RHD was slightly lower than in adjacent populations where rabbits were more abundant. Thus it could be argued that lowered population density reduced the rate of spread of RHDV. There were however distinct regional differences in the patterns of disease outbreaks across the eight experimental sites indicating strong regional influences of climate on epidemiology. Overall, it seems likely that in Australia broad environmental factors are of major importance in driving epidemiology and that rabbit population density (density of susceptible rabbits) plays a lesser role.

Fa *et al* (2001) have added a further complication. In their epidemiological model for RHD they propose that contact rates between rabbits would normally increase with population density with the exception that isolated rabbits move to join other groups of rabbits. This means that the rate of spread of disease may be higher at very low densities than at moderate densities but the rate of spread again increases at very high densities (Fa *et al* 2001, Figure 3 b). It is worth noting that in their model the rate of spread of RHD is lowest at about 25 rabbits/ha whereas only a few Australian rabbit populations such as those at Bacchus Marsh (Victoria) and Turretfield (South Australia) currently reach this density and in many populations where RHD is active, density is in the order of 2-3 adult rabbits/ha.

Virus competition models

White *et al* (2001) developed a model to explain how the presence of a pre-existing non-pathogenic strain of RHDV might effectively prevent virulent RHDV from becoming widely established in Britain. They suggest that differences in rabbit population demography might differentially affect the basic reproductive rates (R_0) of the pathogenic and non-pathogenic strains, leading to each dominating in some populations and not others. Apart from possibly explaining why RHD had a different impact in different parts of Europe, similar models appear useful for considering why virulent RHDV released in 1995 failed to cause significant impact in some parts of Australia despite expectations that rabbits were essentially naïve.

The clear climatic correlates with survival of rabbits as RHD first spread across Australia (Henzell *et al* 2002) may reflect underlying variations in rabbit population demography and possibly different levels of interactions between RHDV and an RHDV-like virus or viruses in the Australian rabbit population.

Nevertheless, it would be unwise to assume that White's model could be used without further modification. In Britain, it has been established that antibodies to non-pathogenic lagoviruses generally protect against acute RHDV (White *et al* 2001) and this is known to be the case for RCV as well (Capucci *et al* 1996) although questionable in some situations (Marchandeau *et al* 2005). In contrast, there is evidence that antibodies to equivalent viruses in rabbits in Australia may not be highly protective (Nagesha *et al* 2000; Cooke *et al* 2002).

It also seems that in some parts of Australia virulent RHDV is not able to spread instantaneously despite rabbits being widely susceptible (Bruce *et al* 2004, Bruce and Twigg 2005). Thus, competing viruses are unlikely to be the only explanation for the failure of RHD to establish.

A predictive model

Despite the development of models to explore and explain the epidemiology of RHD, few of these models are useful for reaching general conclusions or predicting the behaviour or consequences of RHD on a regional or continental scale.

Nonetheless, the climatic variables (temperature, soil moisture) known to limit rabbits in Australia have been incorporated into the CLIMEX population simulation program (Sutherst and Maywald 1985) to produce a model that predicts the distribution of rabbits in Australia. The model has been verified using its ability to closely predict the distribution of rabbits in other parts of the world (e.g. Chile and Tierra del Fuego in South America, North Africa and Europe). The model also reproduces the broad breeding patterns of rabbits in different regions of Australia (Gilbert *et al* 1987) incorporating realistic features such as the shortening of breeding seasons in the arid, sub-tropical and alpine environments and late-season breeding in cooler regions.

The model is also useful for predicting the distribution of European rabbit fleas, *Spilopsyllus cuniculi* (Dale). These are specific parasites of the European rabbit, limited to the more humid parts of the rabbit's distribution in Australia (Cooke 1992). The flea's distribution is constrained not only by those factors that limit the host but also by low humidity that effects development of flea larvae in rabbit nesting burrows (Cooke and Skewes 1988). A further model was subsequently developed to predict the likely distribution of the flea, *Xenopsylla cunicularis* Smit, when it was evaluated for introduction into Australia as an arid-adapted vector to enhance myxomatosis spread in inland areas. In that case, the distribution of the fleas in arid Spain and Morocco was used to set the parameters to predict the fleas' ability to colonize similar Australian climatic regions, although still constrained within the rabbits' distribution because a specific parasite's range cannot exceed that of the host.

Using these same principles, a model has been developed for RHDV anticipating that the virus could occur throughout most of the rabbit's range of distribution in Australia (Cooke 1997). The model has been progressively modified in the light of information such as the lack of virus impact in very wet coastal areas in eastern New South Wales (Richardson *et al* 2007). It is now assumed that conditions for circulation and impact of the virus increase progressively as climate becomes drier, reaching a maximum in semi-arid areas such as the Flinders Ranges area in South Australia (Mutze *et al* 1997). Temperature constraints likely to influence virus behaviour (Smyth *et al* 1997) have also been incorporated into the model.

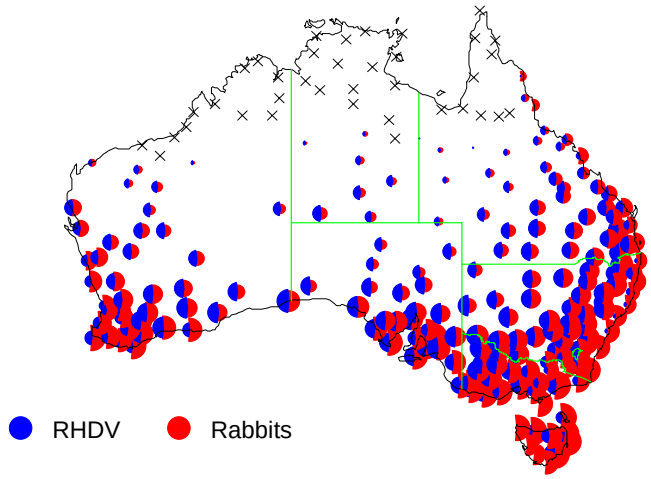


Figure 3. The regional impact of RHDV can be seen by comparing the favourability of the climate at each station for both virus and rabbits. The size of the symbol indicates relative favourability. Crosses indicate that neither rabbits nor virus would persist.

Although developed using data from south-eastern mainland Australia the model is further strengthened by the fact that it confirms that outbreaks of RHD would be most likely in northern and central Tasmania and also in the Hobart region. Likewise, RHD should be more apparent in the semi-arid country and croplands of Western Australia than in the wetter south-west (Bruce *et al* 2004).

The model also provides a picture that generally fits expectations when applied to the European climate data set indicating a decline in impact from southern Europe towards the north and the confinement of obvious outbreaks to south-eastern Britain (Figure 4). A shift in seasonality of outbreaks from "out-breaks associated with the autumn-spring breeding season" in semi-arid Spain (Calvete pers. comm.) to summer outbreaks "at the end of the rabbits breeding season in Britain" (White 2004) also adds a further level of reality to the model's predictions.

Assuming that evolution of resistance to RHD is most likely to be fastest where both disease impact and effective rabbit reproductive capacity are high, data on rabbit productivity and virus impact generated for sites across southern Queensland, NSW,

Victoria and South Australia were combined to indicate that resistance to RHD might be expected to develop most quickly in those parts of Australia where annual average rainfall lies between 300 and 600 mm (Figure 5).

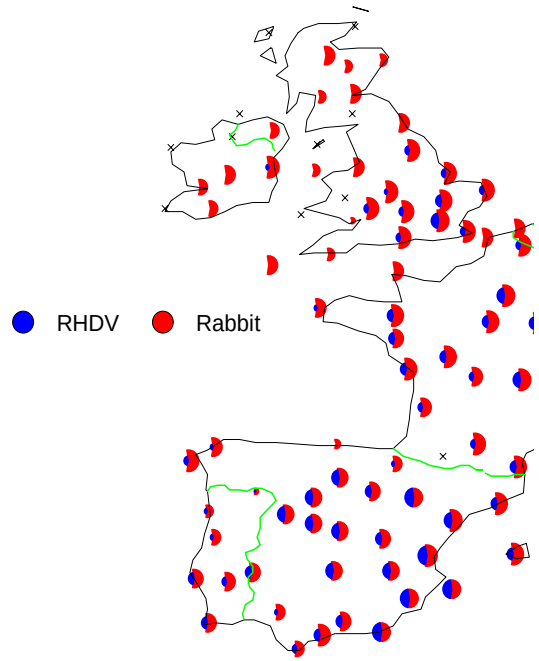


Figure 4. The CLIMEX RHD model based on Australian data predicts that disease impact should be highest in south-east and central Spain and largely confined to south-eastern Britain. This follows the broad patterns observed.

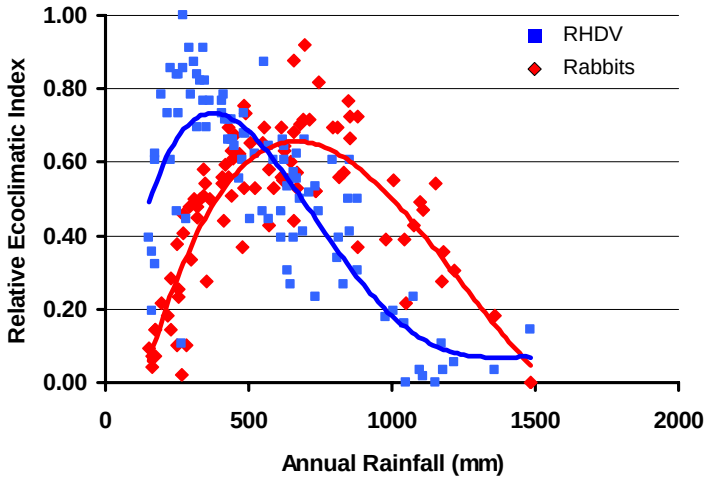


Figure 5. Optimum conditions for both rabbit and virus are found in those parts of south-eastern Australia where rainfall is between 300 - 600 mm annually and it is here that genetic resistance might develop most rapidly.

Appling the same ideas to Europe, the model suggests that genetic resistance would evolve most rapidly in south-eastern Spain in the provinces of Almería, Murcia and

Valencia where RHD has been established longest or near Zaragoza where it was established relatively early and where severe annual outbreaks have been observed. Indeed, Calvete *et al* (2006) have revisited sites in Teruel province, south of Zaragosa, where the impact of RHD was assessed in 1992 and found that 26% of rabbit populations showed evidence of recovery. These populations were characterized as having been severely reduced by the initial outbreaks of RHDV despite being in favourable habitats with light soils They also had rainfall of between 360 and 615 mm annually. Williams *et al* (2006) analysed spotlight count data from 42 sites over a wider area around Zaragosa and similarly found evidence that rabbits were increasing in areas of light soils but found no association with rainfall. Rather, hunting pressure seemed to be keeping populations low. As a consequence it is still unclear whether rabbits in this area are increasing in the way that some Australian rabbit populations appear to be recovering from RHD.

CLIMEX models are not supported by statistical analysis but nevertheless provide a useful, alternative approach and fill a gap in providing predictive models on which to explore new ideas and make future management decisions. As Smyth *et al* (1997) demonstrated, there are several systems for handling and analysing the information required to build more precise models.

Nonetheless, there are important questions that arise from the apparently ready ability of the model developed for Australia to explain the epidemiological situation in Europe. Antibodies raised against the RHDV-like viruses in Australia seem at best weakly protective against RHDV whereas those in Britain are fully protective. On that basis the modelling outcomes need to be treated cautiously until more is known about RHDV-like viruses and their interactions with RHDV in both Europe and Australia.

In Australia the development of genetic resistance to RHD seems most likely in the tablelands and croplands of central NSW, parts of central Victoria and the north-west Wimmera and Mallee regions as well as a large area covering croplands and adjoining pastoral areas in South Australia. It is in these areas that rabbits are likely to show the most rapid resurgence, particularly in areas of lighter sandy soils where warrens can be quickly expanded or renovated.

Summarizing epidemiological data

Despite the huge amount of information collated on RHD, one important task remains. The information still needs to be brought together in a way that provides the most likely picture of how the disease operates and the key factors which will determine its behaviour in the future.

As an example, starting with the analysis of Smyth *et al* (1997) who showed that RHDV spreads most easily when daily temperature ranges between 11ºC and 24ºC, it is worth taking a closer look at temperature, known to influence many aspects of epidemiology, to see if any general conclusions can be drawn.

For well studied sites, such as Gum Creek in South Australia, good information is available on the broad seasonal and diurnal temperature variations of the site and seasonal variations of rabbit burrow microclimate have also been measured (Cooke 1990a). The temperature range of activity for virus vectors such as blow flies and the

shifts in their activity patterns and species composition across the seasons is also known (Norris 1996).

From the perspective of rabbit biology, it is known that temperatures below 10ºC limit pasture growth, while frosting of pastures further reduces its nutritional qualities and rabbits breed poorly under those circumstances. On the other hand there is ample physiological evidence that rabbits suffer reproductive stress once ambient temperatures exceed 27ºC (Cooke 1977).

It is further known that the course of RHDV infection does not change with the ambient temperature over the range from 13ºC to 27ºC (Cooke and Berman 2000) and it is also known that virus survival is high at very low temperatures but falls away quickly with increasing temperature, surviving for less than a day at 40ºC. In this context, Barlow (1999) has argued that to maintain itself RHDV must have a half-life of about 16 days in the environment outside the infected rabbit.

This information, summarized schematically in the figure below, indicates that the minimum temperature allowing rapid virus spread, about 11ºC, is most likely to be explained in terms of temperature limiting blow-fly activity or linked to the minimum conditions for pasture growth and resultant changes in rabbit behaviour in preparation for breeding. By contrast, the maximum temperature favouring virus spread, 24ºC, is more likely to be a consequence of declining virus survival rather than poor vector activity or the cessation of rabbit breeding.

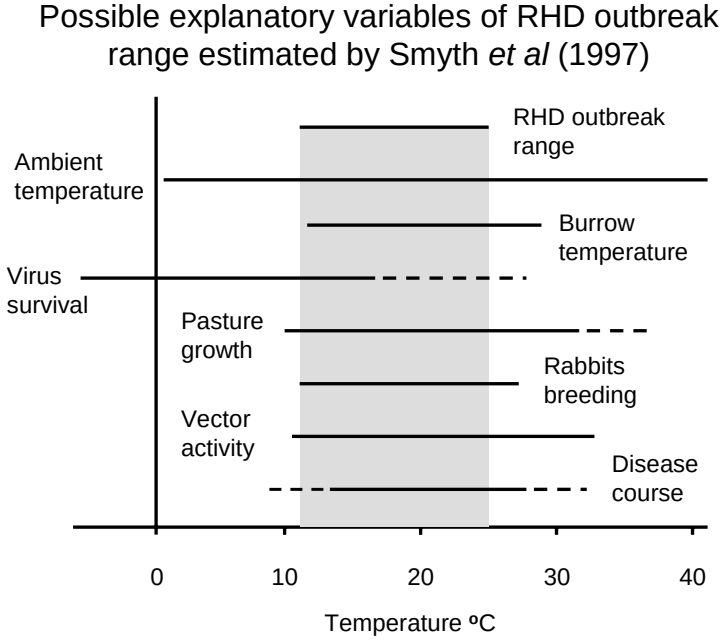


Figure 6. Annual temperature ranges of variables likely to influence virus survival and infection suggesting that poor virus survival may curtail spread at Gum Creek as temperature increases.

An approximate curve derived from published information on virus survival at different temperatures (see *Virus persistence in the environment*) suggests that RHDV

might survive in the order of 128 days at 11ºC but only 14 days at 24ºC (see Figure 7). This rapid decline in survival time appears to be the most likely factor that would limit the chances of spread of the virus from sources such as a dead rabbits. Presumably, the survival of virus in flies would also be curtailed by high temperatures especially since in blowflies flight can raise thorax temperature 4ºC above ambient temperatures (Stavenga *et al* 1993).

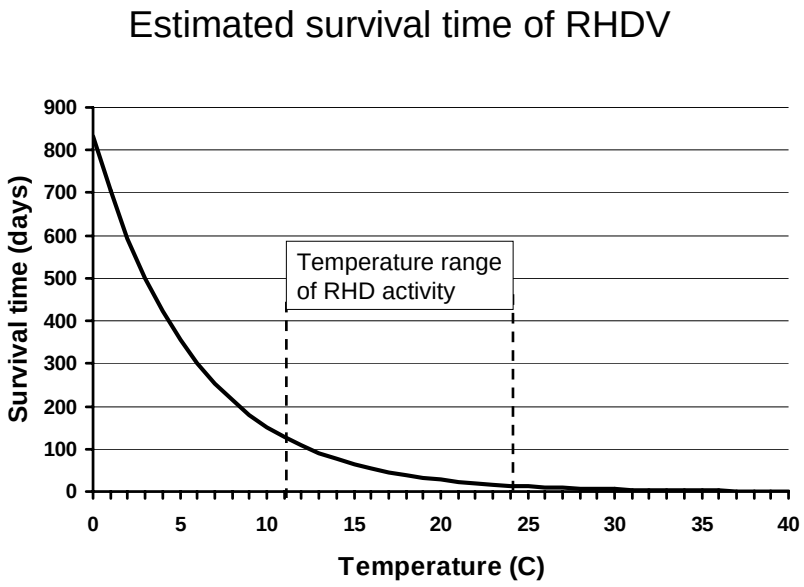


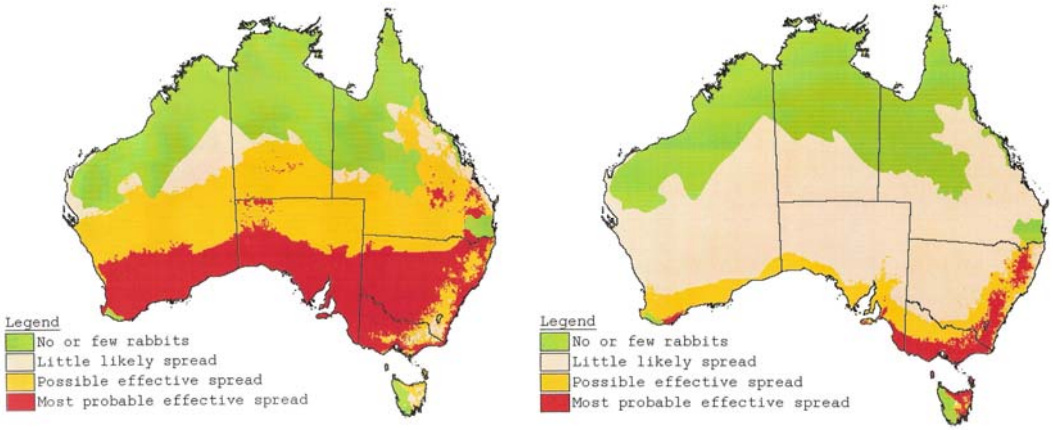
Figure 7. Estimated survival time of RHDV in relation to temperature based on the published information of Rodak *et al* (1991), Westbury (1996), McColl *et al* (2002) and Henning (2003). The temperature range of optimal RHD activity is from Smyth *et al* (1997).

Although no final conclusions can be drawn, putting data together in this way enables some clarification of epidemiology. If a reservoir of virus is necessary for persistence then its most likely form is a rabbit carcase, sheltered from the extremes of daily maximum temperatures within a rabbit burrow. The chances of infection from such a source are heightened when rabbits begin breeding activities, such as investigating and renovating disused warrens, and when vector activity increases. At the peak of summer, even within the relatively protected warren, temperature might increase and virus survival might fall to a level where effective transmission is no longer possible.

These kinds of inferential analyses would be worth further exploration. Concepts developed in one area might be tested indirectly using quite independent lines of argument. For example, the fact that mortality rates from RHDV remain high despite rabbits showing some selection for resistance to infection is entirely consistent with the idea that rabbit cadavers provide an important virus reservoir. It implies that viruses that kill rabbits have the best chance of persisting.

Similarly, from the natural rate of spread following its initial introduction (Kovaliski 1998), RHD could be readily maintained in south-eastern Australia even if high summer temperatures in inland areas curtailed virus survival. Summer temperatures in southern and central Victoria and the tablelands of NSW are low enough to enable

major summer epizootics (Smyth *et al* 1998; K. Butler, H.-J. Yoon and S. McPhee unpublished) and spread of the virus by flies would enable re-colonization and persistence on a regional scale. Climatically, south-western Australia is not as favourable as south-eastern Australia for the circulation and persistence of RHDV over summer (Figure 8).



(a) (b)

Figure 8. Areas where (a) spring and (b) summer RHD outbreaks would be favoured in Australia (reproduced from Smyth *et al* 1997).

Benefits and costs of the release of RHD

Following the spread of RHD across Australia an internal report was produced within CSIRO (Dr S. Ryan pers. comm.) which analysed the environmental, economic and social costs and benefits of the introduction. It was concluded that the environmental benefits were very high as the areas receiving most benefit had been subject to serious degradation by overgrazing but subsequently showed a significant improvement in ecosystem health. Economic outcomes were also highly significant with a net benefit of A$106m annually (0.35% of gross agricultural production) and the net social benefits were high, benefiting some 2,500 pastoral holdings (1.2% of the rural population).

Dr Ryan's review was comprehensive, including consideration of reduced soil loss, increased carbon storage, increased methane production from livestock, R&D costs, impact on pet rabbits and the farmed rabbit industry (e.g. immunization costs) and reduced costs of rabbit control as well as benefits for tourism and impact on indigenous communities. Saunders *et al* (2002) have since listed their observations and provided comprehensive case studies that generally support the CSIRO study in terms of broad economic and environmental benefits. Nevertheless, additional information that continues to become available needs to be included as follows.

Social

There have been no immediate adverse outcomes from the release of RHDV. In the decade since release, it has not been detected in other species nor has the virus 'mutated' rapidly as Smith (1998) suggested. Such ideas have little in common with

current understanding of epidemiological processes in wild animal populations and virus-host co-evolution.

At the time of the escape of RHD from Wardang Island articles in the press, engendered partly by critical comment from Smith (1998), led many people to think that the plan by Australian scientists to use the virus was poorly researched and poorly applied. Certainly, misjudgements were made about the adequacy of Wardang Island as a suitable quarantine area but in general the experimental work showing that the virus would affect only rabbits has been vindicated.

Furthermore, Landström (2001) has argued that the process followed by the Australian and New Zealand Rabbit Calicivirus Disease program (ANZRCDP) was a good example of how an essentially scientific program was effectively presented to the public in both social and cultural contexts. It is important to learn from this experience if the release of further agents for the biological control of rabbits is to be contemplated

If anything, the success of the project brought about its own long-term social impact. Since RHDV spread there has been a general lowering of interest in rabbit damage and economic costs and a subsequent loss of skills to assess the situation and maintain rabbits at very low numbers. This is now becoming noticeable as rabbits begin to recover their numbers once again and it will be essential to re-establish both awareness of rabbit problems and the ability to deal with them to maintain the outcomes achieved.

Economic

Vere *et al* (2004) reviewed previous estimates of the costs of rabbits to Australian agricultural production and the limitations on such estimates brought about by lack of adequate information on costs and benefits. They also noted that environmental costs have not been evaluated. Nevertheless, they were able to estimate the costs of rabbits in Australia's temperate pasture systems and the benefits of reducing rabbits by the introduction of RHD. They concluded that rabbits imposed annual costs on wool producers of between A$7.1 and 38.7 million, depending on rabbit abundance, and that using RHD to control rabbits generated substantial long-term benefits by reducing grazing competition. For example, a 50% reduction in rabbit costs increased the 15 year net present values (NPVs) by A$36.9 and 202.4 million (A$11.8 – 64.9/km^2/yr) nearly all of which was returned to producers. This would have produced a cost-benefit ratio of between 3:1 and 16:1 had the total cost of the program to introduce RHD been met by those producers.

At the Mungerannie Field Day in 2002, cattle producers from inland South Australia were interviewed and asked what the release of RHD and the consequent 90% reduction in rabbit abundance had meant in terms of vegetation and livestock production on their properties (B. Cooke unpublished). Producers were able to provide figures on the annual financial benefits to their enterprises, and these ranged from A$60,000 to $100,000 for individual properties. Correcting this information for property size provided an estimated net benefit that averaged $43.0/km^2/year. Cattle stations in rabbit prone parts of Northern Territory, South Australia and western NSW

occupy a total area of about 600,000 km^2 and so the estimated net annual benefit from introducing RHD was in the order of A$25 million. Over the last 12 years, since RHD became established, this would have amounted to about $300 million in additional net income for those cattle producers.

Ferraro and Burnside (2001) considered the value of public investment in helping landholders to reduce rabbits in western NSW as part of the 'West 2000' program. They concluded that the calculated investment of $2.70 per hectare in rabbit removal would need to be rewarded by an increase in stocking rate of about 14 per cent, or an increase in per sheep productivity by the same amount to ensure that the investment is fully returned after 10 years. Assuming this level of 'break-even' productivity increase is attainable the 200,000 hectares that have been made 'rabbit free' would generate an additional $84,000 in income to landholders. Further assuming that 30 per cent of this is returned in taxation, the benefit on the public investment of $0.40 million was calculated at about $25,000 per annum or 6.25 per cent.

Despite the obvious benefits of RHD, McLeod (2004) estimates that rabbits still cost Australian agricultural producers about A$113m annually. However, as for other reports above, little attempt was made to place a monetary cost on the environmental impact of rabbits. Nonetheless, R. Jones (NSW Agriculture) and W. Gong (University of New England) are currently considering the 'replacement' costs of roadside vegetation in mallee areas as an example of the way that environmental impact of rabbits might be added to the tally of economic costs.

Environmental

When RHD was first considered for use as a biological control agent for rabbits, its likely benefits were weighed against some of the likely disadvantages. For example, there was concern about the likely consequences on species such as the wedge-tailed eagle (*Aquila audax*) that has come to rely heavily on rabbits as prey (Newsome *et al* 1997) Likewise, it was considered that, predators such as introduced red foxes and cats might be forced to prey more heavily on rarer native fauna if their main prey declined.

Among reviews carried out at the time, Pech and Hood (1998) modelled the likely consequences and considered that in semi-arid Australia, with frequent out-breaks of RHD, rabbit populations would decline and large eruptions in their numbers would occur infrequently. Fox numbers would also fall and there would be fewer periods when foxes would be numerous relative to remaining rabbits. Under those circumstances there would be potential for increase of alternative native prey.

Despite some reports of low reproductive rates of wedge-tailed eagles, Edwards *et al* (2002) detected no changes in the eagle population in central Australia following the spread of RHD and Olsen (2005) states that a 75% reduction in rabbits at Lake Burrendong did not noticeably reduce the breeding success of eagles. The predictions of Pech and Hood (1998) have not been followed up experimentally in Australia, but fox numbers certainly declined (Robley *et al* 2004) and Cooke and Fenner (2002) reviewed data from Roxby Downs in South Australia suggesting that a shift in the prey of foxes from rabbits to native species was largely off-set by a decline in the abundance of foxes with no evidence of a net change in predation. It now appears to

be the general consensus that there is little evidence of major deleterious changes (Robley *et al* 2004).

In New Zealand, Norbury (2001) concluded that RHD was likely to benefit dry-land skinks because, with reduced numbers of rabbits, there would be more grass cover and less predation enabling populations to stay above critical levels where predators could drive populations to extinction.

Today, there is little doubt that rabbits potentially affect many native plant populations ranging from terrestrial orchids to acacias in arid woodlands. Rabbits also influence wildlife species such as Common Wombats *Vombatus ursinus* (Cooke 1998), Red Kangaroos, *Macropus rufa*, Western Grey kangaroos, *Macropus fuliginosus*, and Euros, *Macropus robustus*, (Mutze *et al* 1998) and native rodents such as the Hopping Mouse, *Notomys alexis* (Read 2003). All of these species increased when rabbits were experimentally removed. It is also reported that barn owls *Tyto alba* have become permanent residents in very arid areas like Roxby Downs instead of appearing only in exceptional seasons. Presumably, they now have reliable sources of prey (Moseby 2002).

Nevertheless, Denham and Auld (2004) concluded that following the spread of RHD and additional rabbit warren ripping in the Kinchega National Park in Western New South Wales, the survival of seedlings and suckers of trees and shrubs was improved but still not adequate for recruitment into the adult tree populations in the long term.

Murdoch (2005) showed that in the Hattah-Kulkyne National Park in north-west Victoria there had been successful recruitment of seedlings of the buloke *Casuarina leuhmannii* after 1996 when RHD arrived. This was the first recruitment since European settlement of the area and was therefore highly significant. Apparently there were several bouts of seedling recruitment between 1996 and 2002 despite below average seasonal conditions. By 2006 the larger seedlings were over 4 m tall.

Initially there was some confusion as to whether the removal of rabbits by RHD or kangaroo culling that was also extended across the Park in 1996 had allowed this recruitment. However, in 2004 -2005, with kangaroos still kept low but rabbits increasing, it became clear that rabbits alone were seriously damaging the younger bulokes. This was confirmed by direct observation of seedlings severed with a chisel-like cut, typical of rabbit rather than kangaroo browsing, as well as gnawing of bark and digging around the stem and roots of the seedlings.

The pine-buloke woodlands in the Hattah-Kulkyne National Park are regarded as a threatened community under the Victorian *Flora and Fauna Guarantee Act, 1988* but even so, officials within Parks Victoria were caught off-guard by the recent up-swing in rabbit abundance and were unable to avert this reversal of regeneration prospects.

Eldridge and Myers (2001) showed that rabbit warrens and the areas around them supported fewer plant species and generally with lower diversity and species richness. Rabbit grazing removed palatable plants in an area radiating out from the warren and unpalatable weeds such as horehound *Marrubium vulgare* came to dominate the warrens. The authors suggested that although warrens may become abandoned, as was

the case following the spread of RHD, their influence on the environment is likely to be long-lived, possibly in the order of 300 – 500 years.

Eldridge (2006a,b) has continued the work and extended observations to the recovery of vegetation on ripped rabbit warrens and found that although it was substantially changed vegetation quality had not resumed the level seen further away from the warren site. The authors suggest that such restoration of the original woodland vegetation after warren ripping is likely to be a very slow process.

Table 2. Diversity of plant species associated with zones around rabbit warrens at Yathong Nature Reserve (after Eldridge and Myers 2001). Introduced species were mainly unpalatable weeds.

Plant Species	Warren mound	Grazed area	Control	Total
Native	8	17	21	**26**
Introduced	9	7	6	**9**
Total	**17**	**24**	**27**	**35**

Although this study was done in a Nature Reserve it is quite clear that rabbits cause similar changes in open grazing land and farmlands.

Prospects for managing RHD

Reducing rabbit density

After the initial spread of RHD, Government agencies in several states sought to consolidate the situation by reducing habitable areas for rabbits so reducing the risk of subsequent resurgence of rabbit populations (West 2000 in NSW, Bunny Buster in Victoria and Rangelands Action Plan in South Australia). Generally, a 50% subsidy towards the cost of control was offered to land managers to undertake active rabbit control such as warren ripping on private land.

In Victoria, seventeen selected sites have been monitored using night-time spotlight transects, shot samples of rabbits, and counts of the number of warren entrances on selected groups of rabbit warrens. The data collected have been used to estimate the effectiveness of warren ripping in reducing rabbit populations as well as considering the long-term advantages of acting while rabbits remained low. Serological data from the shot rabbits are also useful in establishing the frequency of ongoing RHD outbreaks and indicating the proportion of seropositive (immune) rabbits in residual populations (S. McPhee, unpublished report).

Data show that RHD lowered the general rabbit population across Victoria by about 80%. Further, where this was followed up by a good warren ripping program, rabbits have been held at about 7% of the numbers seen before RHD arrived. However, in areas where ripping was judged to be poorly done, rabbits are currently at about 24% of pre-RHD levels.

At sites like Maryborough and Skipton rabbits have been held at very low levels with a low annual cost for fumigating or ripping re-opened warrens. In areas where no action was taken to destroy rabbit warrens rabbits are now regaining numbers.

Two thirds of rabbits shot on the monitored sites (range 37 – 93%) had antibodies to RHD. About half the kittens shot and 80% of adults were immune. This pattern was consistent over a 4 year period inferring that RHD breaks out regularly each year.

These results show that the key to managing rabbits lies in reducing the breeding population to low levels by warren destruction then keeping them down by regular re-checking and treatment of cleared areas. The cost of re-treatment can be relatively low if work is done thoroughly in the first instance. Given that the remaining adult rabbits on most monitored sites were immune to RHD, effort would be better spent on reducing the size of the immune population by using poisons and warren destruction rather than releasing more RHDV.

Mutze and McPhee (unpublished) compared the epidemiology of RHD on adjacent sites of high and low rabbit density and found that, despite strong regional patterns in epidemiology, lowered rabbit density decreased the rate of spread of RHDV. However, population reduction seemed to have an even stronger influence in reducing the spread of RHDV-like viruses. Although not conclusive, this suggests that lowering rabbit populations should not greatly reduce the incidence of RHD but may reduce the prevalence of antibodies to RHDV-like viruses.

The results clearly establish that complementing RHDV by using a combination of well-established methods of rabbit control, such as warren ripping followed by fumigation, it is quite feasible to maintain rabbits at very low levels. This does not cause major reduction in the effectiveness of RHD and may even enhance it to some extent by reducing potential interaction with RHDV-like viruses. Additional rabbit control efforts usually have long-lasting results because rabbit populations still recover from control operations much more slowly than was the case before RHD first spread.

Release of virus

Bruce and Twigg (2005) demonstrated that the release of only seven RHDV-inoculated rabbits was sufficient to initiate a significant outbreak of RHD in a population of largely seronegative rabbits (3% seropositive, 8% equivocal and 89% negative by competition-ELISA). The population was reduced by about 65%, similar to the reduction observed when RHD arrived naturally on the site some three years earlier. However, they recorded that reduced morbidity rates and spatially patchy spread of the virus had offset the higher mortality of infected rabbits, 92% *v*. 72% in the natural outbreak.

Bruce and Twigg (2005) recommended late spring as the best time to release RHD into rabbit populations. At that time the proportion of seronegative rabbits was high, fleas, mosquitoes and flies were at seasonal peaks and kittens likely to survive the disease because of age-related protection have a low probability of persisting on dry summer pastures. Nevertheless, it is very important to fully consider the risk of initiating outbreaks at that time in terms of allowing more young rabbits to recover

from the disease and substantially augment the immune breeding population. Following from the analysis of RHD impact on rabbit populations in south-eastern Australia (Mutze *et al* 2002) releases in autumn or early winter might be better. Any infected young that survived an autumn outbreak would be a very small component of the following year's breeding population and the slower build up in young rabbits during the rest of the breeding season could avert an RHD outbreak in spring. Most late-born young would then lose their age-related resilience and maternal antibodies over summer and then be prone to acute disease the following autumn.

Recent approval for distributing RHDV on baits should facilitate the initiation of epizootics and so should enable the disease to be used even if natural outbreaks do not occur. However, thought should be given to spreading infective baits on a suitable scale, i.e. putting out small quantities of bait at 10 - 15 sites rather than one large site, to off-set problems arising from patchy and uneven spread. It is unlikely that rabbit blood samples could be routinely tested for antibodies to know precisely when to release virus. The best possibilities for this kind of manipulation of disease will lie in knowing the broad patterns of epidemiology in any region and making recommendations on that basis.

Recent evidence of genetic resistance to RHD infection also calls some ideas into question. For example, we know that there is probably enough RHDV applied to bait to infect rabbits orally but it is less clear if flies still play a major role in spreading RHD. Fly-spots contain relatively few viral particles and as the rabbits' resistance to infection increases it seems likely that quantity of Czech strain v351 virus needed to infect a rabbit could exceed the amount normally found in fly regurgita.

New perspectives arising from this review

Epidemiology

There are many general conclusions that can be drawn from this review. As new information accumulates ideas need to be refined and some long-held ideas rejected.

The use of RHDV in Australia and New Zealand as an agent for controlling rabbits has been generally successful in reducing rabbit numbers. The disease has remained confined to rabbits and there have been few negative ecological problems. However, over the last decade, interest in rabbits as a major agricultural pest has declined, putting Australia in a poor strategic position if rabbits begin to build up again.

There is now an enormous, detailed understanding of the structure of the virus, how it binds to cells to infect the rabbit as well as a growing understanding of how it causes disease. An array of diagnostic methods and tools such as PCR have provided innovative ways of understanding the virus despite the inability to grow it in cell culture and study it more closely.

One of the most fascinating aspects of the disease in rabbits is the relative resilience of young rabbits, supplemented by protection conferred by maternal antibodies acquired across the placenta. This resilience to infection appears to be critical for understanding how some rabbit populations have been able to maintain high levels despite repeated disease outbreaks. Even so we do not fully understand what regulates

the spread of RHD at certain times of the year or in certain regions. Consequently, we cannot fully explain why the disease effectively immunizes young rabbits in some instances yet cause high mortality in others. The importance of climate, population structure and rabbit behaviour as factors influencing epidemiology clearly have roles that may be more important than the numbers of susceptible rabbits present in populations (i.e. density-dependent spread of disease). To this we must add the possible immunizing effects of RHDV-like viruses circulating through rabbit populations in some regions of Australia.

The role of insect vectors has been well considered and although apparently important in the initial spread of RHD across Australia, the abundance of flies as a factor driving outbreaks has since been questioned. Mosquitoes, major vectors for myxomatosis, have not been identified as having a significant role in the epidemiology of RHD.

Interestingly, predation has repeatedly been seen as a more important mortality factor than RHD in many studies where both factors have been recorded together. Such data are biased because mortality collars are usually fitted to adult rabbits whereas RHD frequently spreads among young rabbits, but it nevertheless makes it important that RHD should be seen in perspective as one factor among many that can help regulate rabbit populations.

While we are still awaiting the final results of experiments to determine whether or not rabbits are developing genetic resistance to RHD, this may only provide a partial explanation as to why rabbits are beginning to increase once again in some areas of Australia. Furthermore, even if rabbits are developing genetic resistance we will still be unable to predict the future for RHD because we know so little about the significance of genetic changes observed among virus strains circulating in the field. Until extra research is done in this area we will not be able to answer the dual question: “Will rabbit resistance outstrip the virulence of the virus or will the virus and rabbit co-evolve in a biological arms-race with RHDV remaining as a useful biological control agent?”

General patterns are emerging from the many reported epidemiological studies of RHD. The disease commonly breaks out each year in most rabbit populations but in some it only has a major impact every second year or so. In some populations it may even occur very infrequently.

General models have been proposed to predict both the epidemiology of RHD and likely interactions between virulent RHD and related, less virulent viruses. While attractive, they await further testing using well analysed field data. Advanced methods of analyses (e.g. using the program MARK) are now underway and have enabled not only the concise analysis of complex field data on rabbit antibody status and survival but also many parameters that will be useful in predictive models.

Nevertheless, most models deal with concepts that help us understand details of epidemiology rather than providing a framework for developing testable hypotheses or predicting broad geographical patterns in disease behaviour and the co-evolution of rabbit resistance and virus virulence. It would be useful to explore more ways of predicting where genetic resistance to RHD infection might develop fastest so that

strategic decisions could be made and adequate resources found to counter rising rabbit numbers in those areas.

Implications for future rabbit control

The future of RHD

It seems clear that rabbit numbers are increasing in some areas. Monitoring of rabbit populations in the Flinders Ranges, South Australia, and in the Hattah-Kulkyne National Park, Victoria, both show that rabbits are becoming more abundant yet these trends have not been fully explained. At Hattah-Kulkyne for example, rabbits remained low in 2001 and 2002 when rainfall was good but increased from 2003 onwards despite a continuation of years unfavourable for good pasture growth. This corresponded with the initiation of a fox control program but rabbit numbers have also risen in areas where no fox baiting has been implemented albeit at a slower rate. In the Flinders Ranges, rabbit counts also increased sharply after 2002 despite the fact that a fox control program has been in place for many years.

The timing of this resurgence of rabbits may be coincidental or linked to broadly similar weather patterns but it could also be a reflection of the complex population processes that followed the release of the virus and the subsequent development of genetic resistance in the rabbits or attenuation of RHDV.

The lack of any systematic testing for genetic resistance to RHD during the first 10 years that RHD was present in Australia is regretful, especially since Prof. Frank Fenner, who had detailed the development of genetic resistance to myxomatosis (Fenner and Ratcliffe 1965), had recommended a project of this kind. Nevertheless, if genetic resistance is monitored periodically in the future, it may be possible to determine the rate of increase in resistance.

Ultimately, the co-evolution of rabbit resistance and virus virulence or infectivity will determine whether RHDV will remain a powerful biological control agent. If the virus can retain its ability to infect most rabbits, and kills most of those infected, it will persist and continue to hold rabbits below the numbers seen before its introduction. However, it remains to be seen whether any new equilibrium between virus and rabbits will be fully satisfactory for protecting agricultural production. It is clearly inadequate to protect the regeneration of native shrubs in semi-arid areas such as the Victorian Murray Mallee and similar areas of western NSW and South Australia.

Recommendations for action by land managers

For the majority of individual landholders it will be difficult to monitor the activity of RHD (and myxomatosis) in rabbits on their property closely enough to decide whether or not to release more RHDV (or more myxoma virus). That would best be done in conjunction with local pest control authorities who can monitor what is happening over a wider area.

The best strategy for land-managers would be to use well known methods such as poisoning, warren ripping and follow-up fumigation to reduce rabbits to a low level. RHD will add to the value of the work in many areas by slowing the rate of recovery of populations and reducing the frequency with which control measures need to be re-applied.

Indeed, in some parts of Victoria, where rabbits had remained high despite the presence of RHD, McPhee (unpublished) has demonstrated that where effective rabbit control had been applied, rabbits have since stayed low especially where land managers have been vigilant and re-treated the small number of rabbit warrens that re-opened. RHD may no longer be capable of driving populations low, but it still reduces the prospects of populations building up again.

Removal of rabbits, where possible, also has the advantage that individual land-managers are in better control of grazing pressure on their land and will not have future worries about increasing genetic resistance to RHDV among the rabbits.

Rabbit control in very arid inland areas is generally considered uneconomical given the low productivity of the rangelands. However, even this could be viewed afresh given results obtained by D. Berman (unpublished) at Bulloo Downs, Queensland. Warren ripping in drought, within 1 km of water, was sufficient to eliminate rabbits over very wide areas. There are likely to be other 'drought refuges' for rabbits that could be similarly eliminated by strategic action without recourse to ripping thousands of extra warrens. Myers and Parker (1975) demonstrated that during drought rabbits contracted from the sand hills in semi-arid New South Wales but persisted within a nearby stony pediment zone.

Future medium and long-term research needs

In some ways the advent of RHDV has changed the way in which we must tackle the rabbit problem. Where warren ripping to capitalize on RHD has deprived rabbits of extensive, closely spaced warrens to escape from predators and more cover is provided by regenerating shrubs, it is becoming increasingly common for rabbits to live above ground. This means that in some areas where warren ripping was once the preferred method for rabbit control there now needs to be greater reliance on other methods such as poisoning to prevent rabbits from damaging vegetation and re-establishing extensive warrens. Poisoning has never been used extensively in pastoral area or conservation areas but there is increasing interest in such methods for dealing with localized patches of rabbits.

Cooke (1981) showed that poisoning rabbits was the most valuable first step in removing rabbits from among remnant mallee vegetation on roadsides. Accessible warrens were then ripped and finally fumigation was used to close down the few active burrow entrances sited too close to tree trunks for tractor access. These techniques were subsequently applied to remove rabbits from large (1 km^2) experimental sites in the Coorong National Park set up to assess the impact of rabbit competition on native mammalian grazers such as the common wombat and the western grey kangaroo (Cooke, 1998).

Highly effective rabbit control can be achieved in nature reserves using common techniques. During the experiments in the Coorong National Park, '1080' poison on oat bait (0.04% w/w) was used without incident. Even prior to the commencement of the work it was known that western grey kangaroos and mallee fowl have a high tolerance of this toxin and common wombats, which do not have a high tolerance to '1080', came through unscathed because they are primarily grazers with little interest in picking up grains from a thin trail of oats. The body weight of the common wombat also means that it would be hard for them to eat sufficient poisoned grain to ingest a lethal dose of '1080'. Nevertheless, there are important exceptions (such as brush-tailed possums) and attention should be paid to ways of minimizing possible effects on such fauna not only in conservation areas but also on farmland where they are at risk.

Towards a national rabbit management strategy

Rabbits are increasing again some 10 – 12 years after the introduction of RHD. This is most apparent in Mediterranean and semi-arid parts of north-western Victoria and adjacent South Australia especially "mallee" areas with light sandy soils. However, rabbits are also regaining numbers in parts of the Flinders Ranges, the Coorong National Park and agricultural properties in central Victoria and the Southern Tablelands of NSW where no action was taken to eliminate residual rabbits.

These are all areas where RHD breaks out regularly and still causes heavy mortality. They are also areas where rabbits seem to be developing genetic resistance to RHD. In these areas there is little that can be done to enhance or facilitate the spread of RHD and so the best options for rabbit control are standard procedures such as poisoning, warren ripping and fumigation.

In other areas where RHD breaks out less frequently, genetic resistance to RHD does not appear to be high and it may be possible to initiate new disease outbreaks by releasing virus, either on carrot bait or by intramuscular inoculation of captured rabbits. Nevertheless, this should only be done where other options such as the use of poisons are limited or there is a chance of re-setting the timing of disease outbreaks to a more suitable time of the year. It is rare to find fully susceptible populations and it is inevitable that older immune rabbits will survive any new RHD outbreak.

In the short term, Federal, State and regional organizations with responsibility for rabbit control must be made aware that a resurgence in rabbits is under way in specific areas and that within such areas resources should be provided to suppress rabbits now rather than waiting for the problem to become unmanageable once again. This holding pattern will need to be maintained for at least the next decade or so because that is the time required to explore and introduce further biological control agents.

RHD has provided a natural experiment and given major insight into the devastating impact of rabbits on Australia's native vegetation. As such it has set new standards for rabbit control and if we are going to safeguard native vegetation in many National Parks and within rural landscapes it should be understood that a high level of rabbit control should be the goal. This means keeping rabbits to less than 1 rabbit/ha.

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