

**Factors influencing the epidemiology
of *Rabbit haemorrhagic disease virus*
in New Zealand**

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Jörg Henning

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Abstract

The European rabbit (*Oryctolagus cuniculus*) is a major pest species in New Zealand. The illegal introduction of *Rabbit haemorrhagic disease virus* (RHDV) in 1997, for purposes of biological control of pest rabbit populations, was a controversial event due to uncertainties about the impact of the disease on both rabbit populations and other potential host species. This thesis presents a series of studies conducted to investigate several aspects of the epidemiology and biology of RHDV in New Zealand, and to assess the opinions of farmers about the usefulness of RHDV for rabbit control.

A longitudinal study was conducted in an area of low rabbit density near Himatangi in the lower North Island of New Zealand. Rabbits were trapped at weekly intervals over 37 months using a capture-mark-recapture approach. The study was initiated shortly after the first rabbit haemorrhagic disease (RHD) epidemic occurred in the area, and focused on evaluating the relationships between the occurrence of RHD and the dynamics of the rabbit population. Over the course of the study, predation, particularly by cats, was the principal cause of rabbit mortality. RHDV was present every year in the late summer-autumn period, but caused discernible outbreaks with high mortality only in the first and third years. Fluctuations in population immunity due to population turnover and influx of susceptible immigrants appeared to be key factors contributing to the intermittent occurrence of the disease. Infected migrant rabbits may also be a source of reintroduction of virus and new disease outbreaks. Rabbit deaths due to RHD were clustered in time and space, and RHD affected animals died closer to their home range centroid than rabbits dying of other causes.

In cats, ferrets, stoats and hedgehogs that were trapped at the site, seropositivity to RHDV was detected up to several months before and after RHDV infections in wild rabbits. These predatory and scavenging species may act as vectors causing localized spread of the disease. During the course of the study, the abundance of six fly species identified as potential RHD vectors was also determined. The influence of climatic factors on fly abundance varied between species, and peaks in fly abundance in late summer and autumn coincided with RHD outbreaks.

Two aspects of the survival of RHDV in the environment were investigated using experimental exposure of laboratory rabbits to determine viral infectivity. The survival of RHDV on two matrices (liver and cotton) exposed to environmental conditions on open pasture was evaluated. RHDV in bovine liver tissue, used to emulate a rabbit carcass, remained infective for up to three months under field environmental conditions. RHDV on cotton, which was used to emulate excreted RHDV on an inanimate substrate, remained infective for less than half that time. These observations suggest that RHDV in decomposing rabbit carcasses could be a relatively persistent reservoir of the virus. RHDV that was inactivated with UV-light failed to induce protective immunity in rabbits following oral or parenteral injection, indicating that inactivated virus on baits is unlikely to induce protective immunity in wild rabbits and thereby jeopardise the effectiveness of RHDV use.

Using a multistage sampling frame, the attitudes and practices of farmers regarding rabbit control, and particularly RHDV, were evaluated using a mail questionnaire. Shooting remains the predominant method that farmers use to control rabbits, although 10% of farmers used RHDV baiting. The use of poisoning and trapping for rabbit control has declined since the introduction of RHDV. Most farmers considered that the introduction of RHDV has been beneficial.

The impact of RHDV on rabbit populations appears to be highly variable. These studies have provided detailed documentation on the occurrence of RHDV and its relationship to rabbit population dynamics in an area of low rabbit density. Overall, the findings suggest that both the expected benefits and the potential ecological risks from introducing RHD to New Zealand, were overstated. While the disease certainly had a marked impact on the population at Himatangi at certain times, outbreaks were intermittent and other 'natural' causes of mortality may exert greater constraints on rabbit populations. Better understanding of the factors that contribute to the variability in frequency and severity of RHD outbreaks may enable more efficient use of this method in the future. RHDV is likely to remain a useful option for rabbit control, particularly in areas of severe rabbit proneness, and will likely prove most effective when used in conjunction with other methods.

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'Writing in the speed of thoughts remains the dream of an author' (Heiner Müller, East-German play writer in 'Krieg ohne Schlacht', 1999, Kiepenheuer and Witsch, Köln, Germany), and thoughts need nutrients to flourish. I also found strength and joy for my research in the hours I spent away from it, on the top of some mountains or in remote rugged patches of native bush, and must therefore somehow acknowledge the New Zealand wilderness.

Jörg Henning

EpiCentre, Institute of Veterinary, Animal and Biomedical Sciences

Massey University, Palmerston North, New Zealand

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Introduction

Man has successfully domesticated only a few mammalian species as sources of food and fibre, for transport and labour, and for recreation and companionship. Among these species, the European rabbit (*Oryctolagus cuniculus*) has arguably the most complex relationship with our species, being either revered or maligned according to the circumstances. Originating from the Iberian Peninsula, the territories occupied by rabbits expanded throughout most of Europe, and subsequently to all continents other than Antarctica, facilitated by traders and early settlers, sailors and hunters. The rabbit is farmed for meat and fur production and is an important game species, especially in Europe. It also plays vital roles in some ecosystems, either as stable prey for endangered predator species or by contributing to seed dispersal (Staniforth and Cavers 1977; Palomares 2001). In contrast, the introduction of rabbits into favourable environments, particularly in New Zealand and Australia, has had undesirable outcomes for local ecology and agriculture. Rabbits compete with livestock for grazing, damage the soil surface, destroy crops and seedlings and pose a threat to the ecology through heavy grazing on native plants (Gibb and Williams 1994). In New Zealand, the direct costs of rabbit control in 1995 were estimated to be NZ\$ 22 million (Parkes 1995). The principal means of control are shooting and poisoning, with efforts at biological control in the early 1950s using myxomatosis being unsuccessful (Filmer 1953).

An apparently novel infectious disease of rabbits, now known as rabbit haemorrhagic disease (RHD), was first recognised in China in 1984. A number of general reviews on the biology of rabbit haemorrhagic disease (RHD) have been published in recent years (Ohlinger *et al.* 1993; Thiel and König 1999; Anon. 1996; Cooke and Fenner 2002). The high morbidity and mortality of RHD observed in domesticated and wild rabbits heralded the significance of this disease as both a threat to desirable rabbit populations, and a potential tool for biological control of pest rabbit populations. In contrast with the low public acceptance of myxomatosis in New Zealand in the 1950s (Gibb and Williams 1994), in the mid 1990s public recognition and acceptance of the potential of RHD as a means for rabbit control was considerably stronger (Fitzgerald *et al.* 1996; Wilkinson and Fitzgerald 1998). However, after thorough investigation into the anticipated benefits and risks of introducing *Rabbit haemorrhagic disease virus* (RHDV), the New Zealand government denied an application to import the virus. The virus was subsequently released illegally in 1997 (O'Hara 1997), and is now widely established in this country. However, many aspects of RHD epidemiology in New

Zealand, including temporal and spatial patterns of occurrence and its impact on rabbit populations, remain uncertain.

This thesis reports several studies conducted to investigate aspects of the epidemiology of RHD in an area of low rabbit density in New Zealand, and to evaluate farmers' perceptions on the role of RHD in rabbit control. The reports on these studies are preceded by a literature review on RHDV infection in rabbits. The literature relevant to each of the specific research areas is reviewed in the introduction of each chapter.

In detail, the thesis presented is set out in the following chapters:

Chapter 1: Literature review on the existing knowledge about the agent causing RHD, including characteristics of the disease and its epidemiology in wild rabbit populations.

Chapter 2: Longitudinal study of RHDV infection and rabbit population dynamics in a wild rabbit population.

Chapter 3: Longitudinal study of spatial and temporal patterns of mortality due to RHDV infection and other causes of death in wild rabbits.

Chapter 4: Longitudinal study on temporal association between RHDV infection in wild rabbits and seropositivity in rabbit predators and scavengers.

Chapter 5: Longitudinal study of the influence of weather conditions on abundance of blowflies and flesh flies most likely to be involved in the transmission of RHDV.

Chapter 6: Experimental study of the persistence of RHDV in the environment.

Chapter 7: Experimental study of immune responses of rabbits inoculated orally and intramuscularly with RHDV inactivated with UV light.

Chapter 8: Survey of farmers' attitudes to the use of RHDV and other rabbit control methods.

Chapter 9: General discussion of the results, implications for further research and the use of RHDV as a biological control agent in the future.

Data collection for all longitudinal studies (Chapter 2-Chapter 5) was conducted on a site near Himatangi in the North Island of New Zealand. Experimental studies on rabbits were conducted at Massey University, Palmerston North. Animal ethics approval was obtained for all research involving live animals.

Chapters 2-8 are manuscripts of papers submitted to peer reviewed journals and are presented in the style required by the journal. All papers were submitted before the

submission of the thesis and the journal title is given on the title page of the relevant chapter. Tables, figures and appendices are numbered sequentially throughout the thesis. References and appendices are given at the end of each chapter. The taxonomy for the names and abbreviations of viruses described in the research results of this thesis are according to the Seventh Report of the International Committee on Taxonomy of Viruses (van Regenmortel *et al.* 2000).

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Chapter 1

Rabbit haemorrhagic disease: A literature review

The emergence of rabbit haemorrhagic disease

The first report of an apparently novel infectious disease, now generally known as rabbit haemorrhagic disease (RHD), came from the Jiangsu Province, in the People's Republic of China in 1984 (Liu *et al.* 1984). In a relatively short period of time, this peracute infectious disease of rabbits was recognised in several countries and given numerous names prior to the identification of the causative agent. These included 'X-disease' or 'Mallatia X' (Cancellotti and Renzi 1991), 'Rabbit viral haemorrhagic disease' (Liu *et al.* 1984), 'Viral haemorrhagic disease of rabbits' (Xu *et al.* 1985), 'Rabbit viral sudden death' (Lee and Park 1987), 'Picornavirus haemorrhagic fever in rabbits' (An *et al.* 1988), 'Infectious Necrotising Hepatitis of Rabbits' (Boujon *et al.* 1989), 'Viral haemorrhagic pneumonia in rabbits' (Cao *et al.* 1986) and 'Infectious haemorrhagic disease of rabbits' (Lölinger *et al.* 1989).

In common with all apparently novel infectious diseases in man and animals, the origin of the causative agent has been the subject of considerable interest and speculation. The location of the index outbreak in China clearly suggests a Chinese origin of the disease, and the subsequent spread to Europe has been attributed to exports of rabbits and rabbit meat from China to European states (Haas and Thiel 1993). However, other authors have questioned this explanation, as RHD outbreaks occurred simultaneously in 1988 in several European countries spanning a long distance from eastern to western and southern Europe (Lölinger and Eskens 1991).

Although first recognised in China, these initial cases of RHD occurred in Angora rabbits that had been imported from Germany a few days before the outbreak, and a European origin of the agent has been postulated (Xu *et al.* 1988; Lölinger and Eskens 1991; Xu 1991). It was later suggested, with some support from serological data (Rodak *et al.* 1990a), that a non-pathogenic calicivirus possibly present in Europe for years, developed into a pathogenic strain in the different environment of China (Haas and Thiel 1993).

Some authors saw a possible connection between RHD and a clinically and pathologically similar disease in the European brown hare population (termed European Brown Hare Syndrome - EBHS). EBHS was recognised prior to the emergence of RHD and occurred in hare populations in geographical areas of Europe similar to those

affected by RHD. In addition, experimental studies indicated that cross-infection of these viruses in the heterologous species was possible (di Modugno and Nasti 1990). Genetic studies found a 52% to 60% nucleotide homology between the viruses causing RHD and EBHS, indicating that they are distinct caliciviruses, albeit more closely related to each other than to other known caliciviruses (Nowotny *et al.* 1997).

A feature of RHD has been its rapid dissemination after its initial occurrence in a region. In China, RHD swept over an area of 50,000 km² in less than nine months (Xu 1991). In response to this an eradication program was implemented that included the development of vaccine from inactivated virus, and by the end of 1986 the disease was gradually coming under control (Huang 1991). In 1986, the first cases of RHD were reported from the Republic of Korea (An *et al.* 1988; Park *et al.* 1991). The disease spread in a westerly direction towards Europe, affecting many countries on the way. Outbreaks occurred in the former USSR in 1986 and 1987 and the first clinical cases in Europe were reported in Italy, also in 1986 (Morisse *et al.* 1991). Rabbit meat is an important food in Europe, therefore most of the early reports focused on farmed rabbits. Trade in contaminated rabbit products is suspected to have been the source of many outbreaks (Morisse *et al.* 1991).

In former Czechoslovakia, morbidity and mortality from RHD outbreaks were estimated to be between 80-100%. The spread of the disease was very fast and approximately 30 million animals died in the summer and autumn of 1987 in this country alone (Rodak *et al.* 1991). The first outbreaks of RHD in Germany were recorded in the second half of 1988 (Soike *et al.* 1989; Löliger *et al.* 1989), with mortality varying from 5 to 100% (Maess and Matthes 1990; Schlüter *et al.* 1990a). In common with observations in Austria (Nowotny *et al.* 1992) and in Czechoslovakia (Rodak *et al.* 1991), the affected farms were typically small (Löliger and Eskens 1991). It was suggested that small rabbitries may use more fresh grass or other green feed, which was potentially contaminated by virus from wild rabbits, while large farms prefer industrial meshed feed (Schlüter and Schirrmeyer 1991; Löliger and Eskens 1991).

RHD outbreaks were reported from most continental European countries between 1987 and 1990 (Morisse *et al.* 1991). In 1992, the first RHD outbreak in the United Kingdom was reported in Ascot. One of the outbreaks in the United Kingdom was traced to a shipment of frozen rabbit meat from China, but later transmission

experiments from the same Chinese meat source failed (Chasey 1994). All other outbreaks occurred over a wide area of southeast England, which suggested RHDV had crossed the British channel via ferry traffic, aerosols or birds.

The source for the first outbreak in Mexico City was 18 tonnes of Chinese rabbit meat imported through the United States (Gregg *et al.* 1991). The outbreak was traced to a colony of rabbits owned by a commercial rabbit breeder, who had contact with the frozen meat six days before the first case was reported. These circumstantial links were later supported by phylogenetic studies indicating a close relationship between the Chinese and the Mexican viruses (Nowotny *et al.* 1997). Successful eradication of RHD from Mexico was achieved without vaccination by destroying 110,000 domestic rabbits (Gregg *et al.* 1991), and was undoubtedly aided by the absence of wild European rabbits in this country.

Reunion Island also regularly imports frozen rabbit meat from China. The breakdown of refrigerating units following a cyclone in 1989 resulted in large numbers of spoilt rabbit carcasses having to be disposed. It had been suggested that farmed rabbit colonies on the island became contaminated after dogs had contact with the Chinese rabbit carcasses (Morisse *et al.* 1991). Other non-European countries reporting RHD outbreaks were Egypt (1988), Lebanon (1989), Tunisia (1989), Israel (1990), India (1990) and Cuba (1993). More recent RHD epidemics occurred in 1995 in Benin (Kpodekon and Alogninouwa 1998), in 1996 in Saudi Arabia (Elzein and Al-Afaleq 1999) and in 2000 in the mid-west of the USA (Anon. 2000).

In contrast to many countries, where rabbit meat is considered a valuable protein source, in New Zealand and Australia wild rabbits are viewed as major pests for agriculture and an ecological threat to native fauna and flora. The high morbidity and mortality observed in European outbreaks of RHD pointed to the potential application of the *Rabbit haemorrhagic disease virus* (RHDV) as a biological control agent for pest rabbit populations. Studies to examine the potential of RHDV as a biocontrol agent were underway in Australia when, in 1995, the agent escaped from Wardang Island, a small island 4 km offshore from mainland Australia, and spread rapidly across the continent (Cooke and Fenner 2002). RHDV was illegally introduced in August 1997 to New Zealand, following a rejection by the Ministry of Agriculture to import RHDV as a

means of wild rabbit control (Thompson and Clark 1997). Subsequently, the release of RHDV to control rabbits was legalised and a commercial RHDV product became available.

RHDV taxonomy and molecular biology

Although Liu *et al.* (1984) identified the causative agent of RHD to be viral, its taxonomic status remained uncertain for several years. Early reports classified the agent variously as a picornavirus (Pu *et al.* 1985; Cao *et al.* 1986; Lee and Park 1987) a parvovirus (Xu *et al.* 1988; Gregg and House 1989) or, because of the larger size of RHDV, a ‘parvo-like’ virus (Du 1990). Ohlinger *et al.* (1989) were the first to classify the RHD virus as a calicivirus. After other research teams confirmed this observation (Soike *et al.* 1989; Smid *et al.* 1989; Granzow *et al.* 1989; Valicek *et al.* 1990; Nowotny *et al.* 1990; Rodak *et al.* 1990b; Erber *et al.* 1991; Park *et al.* 1991) *Rabbit haemorrhagic disease virus* (RHDV) was placed in the family *Caliciviridae* (Moussa *et al.* 1992).

Caliciviruses are grouped in four genera. Two of the genera include viruses causing human gastroenteritis (Norwalk-like and Sapporo-like viruses). The genus *Vesivirus*, includes *Feline calicivirus* (FCV), San Miguel sea lion virus (SMSV) and *Vesicular exanthema of swine virus* (VESV). The genus *Lagovirus* includes *Rabbit Haemorrhagic disease virus* (RHDV) and *European brown hare syndrome virus* (EBHSV) (Green *et al.* 2000).

Estimates of the mean diameter of the virions range from 27 nm (Erber *et al.* 1991) to 40 nm (Ohlinger *et al.* 1989). The virus surface consists of regularly arranged, cup-shaped depressions (Smid *et al.* 1989; Granzow *et al.* 1989; Ohlinger *et al.* 1989, 1990a; Rodak *et al.* 1991; Marcato *et al.* 1991). A single stranded, non-segmented, positive sense RNA genome of 7,437 nucleotides is enclosed within a protein capsid (Meyers *et al.* 1991a, 1991b). The genomic RNA encodes, in its first long open reading frame (ORF1), a large polyprotein of 257 kDa. In addition to the genomic RNA of 7.5 kb, a subgenomic RNA of 2.2 kb, which is colinear with the 3' region of the genome, has been found (Ohlinger *et al.* 1990a). RHDV, in common with other caliciviruses, synthesises the capsid protein (VP60) from translation of both genomic and subgenomic

RNA (Parra *et al.* 1993; Boniotti *et al.* 1994; Wirblich *et al.* 1996; Capucci *et al.* 1996a). This large structural capsid protein has a molecular weight of 60 kDa (Parra and Prieto 1990; Ohlinger *et al.* 1990a). Small amounts of a minor structural protein VP10 are encoded by the second ORF (ORF2) located at the 3' end of the RNA (Meyers *et al.* 1991a; Wirblich *et al.* 1996). Furthermore a virion protein, VPg, is covalently attached to the 5' end of both the genomic and subgenomic RNA (Meyers *et al.* 1991a, 2000; Thiel and König 1999).

A low level of genetic variation between RHD viruses has been described in several studies (Meyers *et al.* 1991a; Milton *et al.* 1992; Boga *et al.* 1994; Guittre *et al.* 1995; Nowotny *et al.* 1997; Asgari *et al.* 1999; Moss *et al.* 2002; Le Gall-Recule *et al.* 2003). Guittre *et al.* (1995) found the degree of nucleotide sequence homology for four geographically distinct strains to be 96%, while Nowotny *et al.* (1997) found the sequence homology to be between 89.4-100% for samples from 17 different countries. These results strongly suggested that all RHDV strains are closely related to each other.

In another study, eight different phylogenetic groups of RHDV have been identified based on capsid sequences of British RHDV samples and published sequences from 9 other countries (Moss *et al.* 2002). European isolates had a maximum difference of 14% from the British samples, but little geographical correlation between individual viruses was found, indicating an efficient dispersion of viruses by vectors and human activities. Likewise no clear clustering of sequences according to their geographical origin was found for Australian samples (Asgari *et al.* 1999).

Little genomic variation among RHD viruses was seen over time between Australian samples obtained over a two-year period (Asgari *et al.* 1999), nor between French isolates collected over 11 years (Le Gall-Recule *et al.* 2003). The Australian study indicated only minor genetic differences (98.2% to 100% identity) in the RHDV sequences from different field samples compared to the original Czechoslovakian strain of the virus (Asgari *et al.* 1999).

In contrast, some studies assessed antigenic variation in RHDV, based on the virus's ability to agglutinate human erythrocytes (Chasey *et al.* 1995; Capucci *et al.* 1996b), while others demonstrated greater variation in the VP60 gene between RHDV strains obtained from vaccinated rabbit flocks in Germany (Schirrmeier *et al.* 1999). A

particularly interesting development in the epidemiology of the disease, is the isolation of a highly pathogenic form of RHDV, which was found in Italy (Capucci *et al.* 1998) and more recently in France (Le Gall-Recule *et al.* 2003).

Non-pathogenic rabbit calicivirus

A non-pathogenic calicivirus, which was named rabbit calicivirus (RCV) by Capucci *et al.* (1996a), was found in domestic rabbits in Europe (Capucci *et al.* 1997). This virus is closely related to RHDV, but has notable differences with regards to pathogenicity, tissue tropism and the primary sequence of the structural protein (Capucci *et al.* 1996a). While various RHDV isolates have shown 98% homology with each other, the non-pathogenic calicivirus has an average of 91.5% amino acid identity with RHDV (Capucci *et al.* 1996a; 1996c). RCV infected rabbits appeared healthy; there was no evidence of histopathological lesions after necroscopy and the highest concentrations of the virus were detected in the intestines (Capucci *et al.* 1996a). Moreover, animals, which were experimentally infected with this virus, seroconverted to RHDV and were protected against challenge with virulent RHDV. In Italy, natural infection with non-pathogenic calicivirus was further demonstrated in a commercial rabbitry, where animals became infected shortly after weaning leading to seroconversion in the absence of clinical disease (Capucci *et al.* 1997). There was up to 19% variation in the nucleotide sequence of the Italian avirulent RCV compared to British viruses (Moss *et al.* 2002).

Serological evidence from Europe points to the existence of non-pathogenic viruses related to RHD preceding the recognition of the disease. Sera from Czechoslovakian laboratory rabbits collected up to 12 years before the detection of RHD in this country, contained antibodies that cross-reacted with RHDV (Rodak *et al.* 1990a, 1991). Similar observations were made in experimental and domestic rabbit populations, where no clinical signs of RHD had ever occurred (Capucci *et al.* 1991; Smid *et al.* 1991; Nowotny *et al.* 1992; Chasey *et al.* 1995). Moss *et al.* (2002) also showed that RNA in sera from healthy domestic rabbits stored for nearly 50 years, and in rabbit sera from healthy wild rabbits collected during the 1990s in Britain, is genetically highly homologous to virulent RHDV circulating in rabbit populations today. These authors concluded that viruses closely related to RHDV had almost certainly circulated

harmlessly in Britain and Europe for centuries prior to the emergence of the disease. Additional evidence consistent with this possibility comes from serological surveys that found antibodies against RHDV in areas where the disease was not fully established. RHDV antibodies were found in wild rabbit sera from areas in both the United Kingdom and Ireland when the disease was not widespread, supporting the view that a non-pathogenic RHD was circulating in these wild rabbit populations (Chasey *et al.* 1997a; Trout *et al.* 1997a).

A low degree of seroreactivity, that did not provide protection against disease, was detected in wild rabbits in Australia prior to the escape of the virus onto the continent (Lenghaus *et al.* 1994). Later, serological investigations confirmed the presence of 'pre-existing' (i.e. prior to the introduction of virulent RHDV) cross-reactive antibodies to RHDV in Australian rabbits (Nagesha *et al.* 2000; Robinson *et al.* 2002a). Similarly, in New Zealand approximately 6% of rabbit sera, collected before the introduction of RHDV, tested positive on a competition Enzyme Linked Immunosorbent Assay (ELISA) (dilution 1:40), which was consistent with the existence of a non-pathogenic calicivirus in this country (O'Keefe *et al.* 1999). However, as most seroreactivity occurs at low dilutions in the competition ELISA, for RHD affected areas differentiation between titres to RHDV and to the putative RHDV-like virus is difficult. Cooke *et al.* (2000; 2002) used a combination of competition ELISA and isotype ELISA titres in an attempt to classify the immunological status of rabbits. The combination of strong reactivity (dilution unspecified) in the IgG ELISA for a rabbit serum, with low titres ($\leq 1:10$) in the competition ELISA, was interpreted as evidence of the rabbit's exposure to an RHDV-like agent. This approach not only allows identification of seroreactivity in 'pre-RHD' sera, but provides a means of discriminating between exposure to non-pathogenic virus and pathogenic RHDV in areas where RHDV is present. However, despite serological evidence, the presence of such non-pathogenic viruses has not been confirmed by virus isolation anywhere other than in Italy.

The ability of seropositivity to RHDV-like viruses to confer protective immunity to RHD appears to be inconsistent. Experimental challenge with RHDV of seropositive domestic and wild rabbits, that had not experienced RHD outbreaks, gave the rabbits effective protection from the disease (Rodak *et al.* 1991; Smid *et al.* 1991; Chasey *et al.* 1995, 1997b). Cross-immunity induced by a benign RHDV-like virus is presumed to be widespread in Britain, and may explain the relatively low impact of RHDV in wild

rabbits in many areas (Trout *et al.* 1997a; Trout 1999). In Australia, 20 wild rabbits with low antibody titres trapped before RHDV spread throughout the country, all succumbed to experimental infection with RHDV (Lenghaus *et al.* 1994), while in a second Australian study only 11 of 23 seropositive rabbits caught in an RHD free area were infected following experimental infection (Nagesha *et al.* 2000). Similarly, only 12 of 60 rabbits obtained from Northland, New Zealand, before the arrival of RHDV, survived experimental challenge with the virus (Parkes *et al.* 2002). Furthermore, correlations between antibody titres prior to challenge and survival following challenge have been inconsistent between studies (Nagesha *et al.* 2000; Cooke *et al.* 2002; Parkes *et al.* 2002). Cooke and Fenner (2002) concluded that Australian rabbits with antibodies to the putative RHDV-like viruses are not fully protected against RHD, and suggested that these agents may be more remotely related to RHDV than non-pathogenic viruses in Europe. The moderate impact of RHD in some high annual rainfall areas in Australia (Henzell *et al.* 2001, 2002) has been noted. More specifically Cooke *et al.* (2002) reported a higher prevalence of antibodies against RHDV-like virus in areas that receive an annual rainfall in excess of 400-500 mm.

Using modelling, White *et al.* (2001) investigated the variable impact of RHDV between areas in Britain, which was explained by the dominance of either pathogenic or the non-pathogenic virus. The authors assumed that the pathogenic virus is highly infectious, but only for a short time, while infection with the non-pathogenic virus results in a life-long persistence associated with population immunity to the pathogenic agent at some sites. However, in further modelling studies, the same authors downplayed the importance of different viruses and emphasised the likely role of acute versus chronic infection with RHDV. Acute infection would result from large viral doses and manifest itself as a short duration, highly infectious disease with high mortality. In contrast, chronic infection would be of longer duration, but with diminished viral shedding and no mortality (White *et al.* 2002). The authors suggested that chronic infection could exclude acute infections in a population, but not the reverse, although the possibility of disease emergence due to immunosuppression of chronically infected animals was raised. However, many of the assumptions in this theoretical work, including the existence of long-term carrier rabbits and dose-response effects associated with chronic or acute states of infection, are yet to be confirmed in the field.

Properties of *Rabbit haemorrhagic disease virus*

The ability of a virus to survive in the environment is a key factor in its epidemiology and control. In common with other caliciviruses, RHDV is a relatively resistant virus. It is resistant to ether and chloroform and can survive in solutions with pH values as low as 3.0 (Xu and Chen 1989; Du 1990; Xu 1991). However, solutions of 1% formalin, 0.5% sodium hydrochloride (10% household bleach), 2% vanadine, 4% calcium hydroxide (whitewash) and 2% 'One Stroke Environ' are all effective disinfectants for RHDV (Gregg *et al.* 1991). Xu (1991) was able to destroy RHDV after incubation of infected liver emulsions at 37°C for 1 hour with both 2% NaOH and 2% formalin solutions. Ohlinger *et al.* (1990a) used a concentration of 0.4% formalin for 24 hours at 37°C to produce inactivated virus for immunisation. In contrast, Smid *et al.* (1989) reported RHDV to be highly resistant to formaldehyde. A recommended disinfection procedure for rabbit cages or sheds should involve a treatment with 10% bleaching powder (or 3% formalin), followed by 2% NaOH with housing then left empty for a minimum of two weeks in summer to up to two months in winter (Xu 1991).

RHDV is also tolerant of broad ranges in temperature. Infected liver samples did not show any loss of infectivity after storage at -5°C for 413 days, at -20°C for 560 days, or at -70°C for 4.5 years (Xu 1991). RHDV also resists heating to 50°C for 60 minutes (Du 1990; Xu 1991). Smid *et al.* (1991) showed that RHDV dried on cloth survived at room temperature (approximately 20°C) for 105 days, although there was some evidence of reduced infectivity. Furthermore, RHDV survived in an organ suspension at 4°C for at least 225 days (maximum time tested) and also retained its infectivity at 60°C for 2 days (maximum time tested) in an organ suspension and when dried on cloth.

Environmental persistence of RHDV was also indicated by RHD mortalities in rabbits introduced into temperature-controlled rooms (22°C) in which RHDV inoculation experiments had been conducted 4 weeks previously (Lenghaus *et al.* 1994). McColl *et al.* (2002a) examined the persistence of RHDV in infected and decomposing rabbit carcasses, also at 22°C (McColl *et al.* 2002a). The death of three susceptible rabbits occurred following their exposure to samples collected after 20 days. However, no deaths resulted from using samples collected after 26 or 30 days of storage, although

seroconversion occurred in some rabbits. Bone marrow from known RHDV-positive rabbits was shown, using the reverse transcriptase polymerase chain reaction (RT-PCR), to contain RHDV RNA after 7 weeks of exposure to the environment (Moss *et al.* 2002). However, the authors did not confirm the viability or infectivity of the virus. Overall, there is a body of evidence that indicates that the ability of RHDV to survive in the environment, and particularly in carcasses, may be an important attribute of the virus. However, the importance of virus survival has not been directly evaluated under field conditions.

Existing knowledge about the survival of the virus in the natural environment is derived from field observations of rabbit populations. Based on the presence of ongoing infection and mortality of rabbits, Marchandeau *et al.* (1998) in France, and Simon *et al.* (1998) in Spain, suggested that RHDV persisted in the environment over periods of several months. Cooke (1999a) combined data on the presence of virus in rabbits and in flies on the Gum Creek Station study site in Australia during 1996 and 1997, and calculated that the longest period that the virus could have been absent from the study site was three months. However, the apparent persistence of RHDV from year to year in the same areas could be attributable to virus shedding by carrier rabbits, or its mechanical spread by vectors or rabbits, rather than the long-term survival of RHDV in the environment (Cooke 2002; Calvete *et al.* 2002).

The potential importance of ambient temperature (Cooke and Fenner 2002) can be inferred from observations that RHDV releases and natural outbreaks are promoted by cooler temperatures in Australia (Kovaliski 1998; Lugton 1999). This evidence was supported by more detailed analysis (Neave 1999; Henzell *et al.* 2002). Rabbit mortality was in general higher in drier areas of inland Australia than in the cooler, wetter areas in eastern Australia (Neave 1999). The same dataset was analysed by comparing rabbit spotlight count data as a measure of rabbit survival with principal components of 35 climate variables (Henzell *et al.* 2001, 2002). This model indicates that a combination of high humidity and high temperatures results in reduced RHD effectiveness in summer. The authors suggested that RHDV inside the rabbit warrens would be inactivated under these conditions in summer. In contrast, preparation of warrens for breeding in autumn would expose RHDV deep in the warren system, which may persist longer in this environment due to lower temperatures and lower humidity, thereby

resulting in RHD outbreaks. The differential persistence of RHDV inside burrows might therefore influence the effectiveness of the disease (Calvete *et al.* 2002). Infected burrows would be a reservoir for new infections, resulting in endemic outbreaks in the breeding season, whereas low RHDV activity in areas where burrows are sparse would lead to an irregular pattern of outbreaks influenced by reintroduction of virus by infected animals. Some authors have raised the possibility that inactivated RHDV on contaminated baits might result in protective immunity of rabbits (O'Keefe *et al.* 1999); the high prevalence and titres of RHDV antibodies associated with high survival rates has occurred in areas where the deliberate release of RHDV baits ('biociding') was used in New Zealand. Although the survival of RHDV in the environment in baits, carcasses and on other matrices may greatly influence the epidemiology of RHD (Cooke 1999a), there is a paucity of data on virus survival under natural conditions (Henzell *et al.* 2002).

Host range of *Rabbit haemorrhagic disease virus*

The potential host range of an infectious agent is another key determinant of the predominant epidemiological patterns of its associated disease. Caliciviruses are widespread in nature and have been isolated from numerous mammalian species including humans, chimpanzees, calves, pigs, cats, mink, skunks, rabbits, hares, sea lions and dolphins. SMSV, VESV and FCV can be grown in tissue culture, while the human caliciviruses, EBHSV and RHDV, cannot be cultured *in vitro* (Lenghaus *et al.* 2000). It has been suggested that culturable caliciviruses in particular could be capable of infecting diverse mammalian hosts leading to the horizontal spread and emergence of new disease syndromes in the secondary host species (Smith and Boyt 1990) and that caliciviruses of marine origin have considerable potential to infect other hosts (Smith *et al.* 1998a).

SMSV, which is able to infect several marine mammals and fish species (Smith *et al.* 1980), can cause vesicular disease in swine (Barlough *et al.* 1986a, 1986b; Berry *et al.* 1990) and humans (Smith *et al.* 1998b). However, the host range of some other caliciviruses appears to be restricted only to the primary host and closely related species (Lenghaus *et al.* 2000). For example, *Norwalk virus* appears to infect only humans and chimpanzees (Wyatt *et al.* 1978), and FCV disease has only been described in *Felidae*

(Smith *et al.* 1998a; Lenghaus *et al.* 2000). Although mutations of FCV have been reported (Seal *et al.* 1993), there is no evidence that this was associated with an expanded host range.

The evaluation of host range is of interest with respect to the possible origin of an apparently novel agent, and to the potential ecological impact that could ensue were the agent introduced into a new environment. Logically, the most likely hosts to share susceptibility to a given virus are the most closely related host species. Epidemics of clinical EBHS in hares preceded RHD epidemics in rabbits by 2-3 years in France (Morisse *et al.* 1991), Italy (Cancellotti and Renzi 1991), Denmark (Henriksen *et al.* 1989), Great Britain (Chasey and Duff 1990) and Germany (Goldhorn 1987; Eskens *et al.* 1987; Eskens and Vollmer 1989; Lölinger and Eskens 1991). On the Swedish island of Gotland, the lag between clinical cases of EBHS and occurrence of RHD was approximately 10 years (Gavier-Widen and Morner 1991). These temporal patterns, together with antigenic and clinical similarities, led to suggestions that RHDV may have arisen from the virus causing European Brown Hare Syndrome (Cancellotti and Renzi 1991).

Experimental infection of hares with RHDV gave variable results (Mocsari 1990; Lölinger 1990; Kölbl *et al.* 1990; Du 1990; di Modugno and Nasti 1990; Ohlinger *et al.* 1990b; Morisse *et al.* 1991; Chasey *et al.* 1992). Cross protection experiments (Nauwynck *et al.* 1993; Lavazza *et al.* 1996; Laurent *et al.* 1997) indicated that EBHS virus does not protect rabbits against RHDV challenge, and *vice versa*. These results were supported by the findings of Capucci *et al.* (1991), Chasey *et al.* (1992) and Nowotny *et al.* (1997), who, by using a range of procedures including nucleotide sequencing, found that there were distinct differences between RHDV and EBHS, and concluded that it was unlikely that RHDV originated from EBHS. However, cross-reactions with monoclonal antibodies to the heterologous virus were reported by Capucci *et al.* (1991). Type specific antibodies appear to react with exposed proteins responsible for haemagglutination and viral infectivity, while monoclonal antibodies react with epitopes on core protein structures. Along with hares, several other lagomorph species including cottontail rabbits (*Sylvagus sp.*), jack rabbits (*Lepus sp.*), and volcano rabbits (*Romerolagus diazzi sp.*) appear not to be susceptible to RHDV (Gregg *et al.* 1991). Intranasal inoculation of RHDV of hares, guinea pigs, mice, golden

hamsters, chinchillas and piglets did not result in clinical signs of RHDV one month post inoculation, but no further serological and virological diagnostic procedures were performed in this experiment (Smid *et al.* 1991). Shien and Lee (2000) were able to detect RHDV RNA in lung and liver tissue up to 14 days post inoculation (p.i.) in subcutaneously infected piglets. However, viral detection was transient and antibody titres were low (peaked at 6 days p.i. at 1:32 dilution) and of short duration, despite the high doses (at least $10^{6.5}$ rabbit LD₅₀) used in the inoculum. The authors inferred from these results that RHDV could be transported from the inoculation site to different organs if a high viral dose was used. Overall, current evidence suggests that the host range of RHDV may be relatively narrow.

In Australia and New Zealand, ecological concerns about introducing RHDV as a biological control agent led to intensive transmission studies in non-target species. Laboratory testing of 28 different domestic, native and feral Australian animals was carried out by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) in a high security Australian Animal Health Laboratory (AAHL) (Lenghaus *et al.* 1994; Gould *et al.* 1997). Four animals of each species were inoculated intramuscularly with 1000 rabbit LD₅₀ and monitored for clinical disease and seroconversion (Lenghaus *et al.* 1994). Pathological examinations were conducted after two weeks and tissue samples were tested for the presence of RHDV (Gould *et al.* 1997). No clinical signs or gross-pathological or histopathological changes were observed and no evidence of viral infection was detected using RT-PCR. The only concerning result was found in mice where three mice developed low antibody titres, but these titres were suggested to be just an immune response to injected RHDV antigen rather than a direct result of viral replication.

In New Zealand the Department of Conservation (DOC) decided to evaluate RHDV susceptibility in two selected native species, which were considered likely to come in contact with forages and burrows used by rabbits. These were the North Island brown kiwi (*Apteryx australis mantelli*) and the lesser short-tailed bat (*Mystacina tuberculata*). Bats are the only native land mammal of New Zealand. Inoculation of the bats did not induce any serological response, while all Kiwis tested seropositive by 14 days post inoculation, with rising titres up to 33 days. No gross or histological lesions of RHDV were observed in two kiwis killed after 48 days and no RHDV RNA was detected. In

the other two birds, antibodies persisted until 136 days post exposure when the study terminated. Again, the serological response was attributed to a response to the viral antigen in the inoculum, which was 300-fold greater than in the CSIRO-AAHL study (Buddle *et al.* 1997).

Smith (1999) questioned the conclusions drawn from the CSIRO-AAHL tests and the inoculation of New Zealand native species. He criticised the standard of laboratory practices and the sampling procedures used and concluded that 11 of these species showed evidence of seroconversion following inoculation.

Some predating and scavenging species will have intimate contact with rabbit carcasses. The possibility that serological responses may develop following consumption of RHDV infected rabbit was confirmed experimentally in six red foxes (*Vulpus vulpus*) (Leighton *et al.* 1995). The foxes were dosed orally with approximately half of an adult rabbit liver (40 g) from rabbits that had succumbed to RHD. Liver homogenate was also painted on all surfaces of the mouth and pharynx. Antibodies were detected 7 days post infection and declined after 14 days, but three foxes retained titres of 1:20 after 176 days. No effort was made to detect RHDV in the exposed foxes, and the occurrence of RHDV replication in this species remains uncertain. Seroconversion in domestic dogs, which were inoculated by intraocular and parenteral routes with RHDV, was also reported, but replication of virus was not assessed (Simon *et al.* 1994). Infective RHDV that caused disease in susceptible rabbits was also found in faeces of dogs which had been fed infected rabbit livers (Simon *et al.* 1994).

A serological survey of 352 red foxes shot in Germany revealed 18 samples (5%) to be seropositive using a blocking ELISA, and eight samples to be positive in the haemagglutination inhibition test (Dedek and Frölich 1997; Frölich *et al.* 1998). In contrast, no antibodies were found in 75 free-ranging foxes in France when the less sensitive haemagglutination inhibition test was used (Simon *et al.* 1994).

Serological tests of 259 humans, who were potentially exposed to RHDV in Australia, did not detect antibodies to RHDV (Carman *et al.* 1998; Carman 1999). In addition a survey of disease symptoms did not find significant differences between exposed and unexposed people, or with subsequent episodes of illness. Again Smith

(1999) and Matson (1999) re-analysed the data and claimed evidence for increased disease incidence, and the outcome remains in dispute (Carman *et al.* 1998; Carman 1999). Before the introduction of RHDV into New Zealand, 495 samples from blood donors living in areas of high rabbit infestation were tested, but no specific antibodies to RHDV were found (Jennings 1999). In two rural communities, no serological evidence of RHD infection in humans was found in 104 participants, including many heavily exposed individuals, tested 4 months after the formal identification of the virus in New Zealand (Greenslade *et al.* 2001). The only anecdotal case of a human developing RHDV antibodies was reported during an RHD eradication program in a high security laboratory in Mexico. A staff member, who had a high antigen exposure, developed antibodies against RHD at a 'low level' over a short time (Bureau of Resource Studies 1996).

The transmission of RHD

Transmission of RHDV can occur via oral (contaminated food, biting), nasal (aerosols), ocular (flies, aerosols) or parenteral (blood drawing insects) routes and may be either direct or indirect. The virus is commonly stated to be present in all secretions and excretions of diseased rabbits (Ohlinger *et al.* 1993), but supportive research about the duration of excretion or secretion is scarce.

Transmission between rabbits

Passage of RHDV from infected liver cells, the major site of viral replication, via bile to the intestines leads to viral excretion in faeces (Morisse *et al.* 1991), and the faecal-oral route is assumed to be the most important transmission method (Gregg *et al.* 1991; Morisse *et al.* 1991; Nowotny *et al.* 1993). Faeces and urine from rabbits that recovered from RHD was infectious for up to one month, but not after 8 weeks following recovery (Gregg *et al.* 1991). Although Collins *et al.* (1996) were unable to detect RHDV antigen in urine and faeces from infected rabbits, Nowotny *et al.* (1993) found the highest concentration of antigen in the bile, followed by faeces (up to 7 days post inoculation) and then urine. In contrast, conjunctival secretions had low and irregular titres. Based on the titres, Nowotny *et al.* (1993) inferred that urine and conjunctival secretions play only a minor role in RHDV transmission. Shien *et al.*

(2000) indicated that RHDV can be gradually cleared from young infected rabbits (4-5 weeks old). Viral RNA in urine and faeces was detected after 36 hours post inoculation, but was not detectable 4 days after inoculation, while virus persisted in bile and spleen for at least 47 days post-inoculation. Some wild rabbits from the South Island of New Zealand, which tested negative for antibodies in the competition ELISA, but had elevated IgG titres, had detectable amounts of RHDV in their livers or intestine (Zheng *et al.* 2002). It was hypothesised that these animals were persistently infected following exposure to sub-lethal doses of RHDV and act as carriers for disease transmission and maintenance.

Direct transmission routes would include oral or conjunctival transmission during grooming or transmission through skin laceration (e.g. from fighting) by subcutaneous, intradermal or intramuscular routes. In addition, oral transmission would occur during the process of coprophagy or indirectly via consumption of contaminated food.

Experimental transmission of RHDV has been achieved via the intramuscular (Schirrmeyer *et al.* 1990; Cooke and Berman 2000; Robinson *et al.* 2002b), intradermal (Cooke and Berman 2000), subcutaneous (Frescura *et al.* 1989a), conjunctival (Gehrmann and Kretzschmar 1991), intranasal (Lölig *et al.* 1989) and oral (Cooke and Berman 2000; Parkes *et al.* 2002) routes. Experimental studies testing the effects of inoculation routes have shown that rabbits exposed by either intradermal or intramuscular routes died approximately 21 hours earlier than animals dosed orally (Cooke and Berman 2000).

Aerosol transmission is unlikely to be an important transmission route, unless rabbit faeces or blood are aerosolised, as may occur during cleaning procedures in rabbitries for example (Gregg *et al.* 1991). This was confirmed in experimental studies, where no infection occurred in control rabbits kept in a fly-free room at 50 cm distant from RHDV inoculated animals (Gehrmann and Kretzschmar 1991). No studies have investigated whether venereal transmission can occur.

Transmission via insects

Transmission routes via insects

Transmission of RHDV by insects has been shown in several studies. A commissioned Ministry of Agriculture and Fisheries policy report on potential vectors of RHD from Crosby and McLennan (1996) classified flies as direct-contact (virus transmission between infected and uninfected rabbit) and indirect contact (virus transmission via rabbit foraging) vectors.

Asgari *et al.* (1998) demonstrated that blowflies (*Calliphora* and *Chrysomya* spp.) retained viable virus for up to nine days after feeding on infected rabbit liver and estimated that infectious flyspots contained 2 to 3 lethal doses_{50%} (LD₅₀) of RHDV. Gehrmann and Kretzschmar (1991) observed that iridescent flies (*Phormia* spp.) transmitted RHDV to susceptible rabbits seven hours after being contaminated with RHDV-infected liver material. These authors proposed that conjunctival contact is the principal route for fly-borne transmission of RHDV, and achieved conjunctival infection of rabbits with approximately 10^2 RHD virus particles.

Studies of RHDV in bush flies and blowflies in Australia and New Zealand (Westbury 1996; Heath *et al.* 1999; McColl *et al.* 2002b) indicate that RHDV can be ingested by flies, and that sufficient virus is present in the environment to contaminate flies externally. Using PCR techniques, RHDV was detected in the bodies of blowflies for several days after feeding on infected liver, but for only 7 hours on fly legs (Asgari *et al.* 1998). The researchers concluded that contamination of pasture with viable virus through fly faeces has to be expected and that this route may be the most likely mechanism of virus transmission from blowflies (Asgari *et al.* 1998).

Transmission of RHDV to rabbits by non-biting flies might occur by direct contact of flies with mucous membranes (eyes, nose, anus), ingestion of herbage contaminated with fly faeces, or through contact with burrow entrances contaminated by flies (Asgari *et al.* 1998; Heath *et al.* 1999). If RHDV is deposited by flies on a rabbit body, grooming of the contaminated area by rabbits will probably be necessary to result in infection (Westbury 1996).

RHDV most likely does not replicate in insects (Westbury 1996). No RHDV virus was detected in adult flies that developed from maggots on RHDV-infected rabbit carcasses; neither infection of internal tissues of flies occurred, and nor was any virus eliminated from fly tissues during metamorphosis (Asgari *et al.* 1998).

Variability in the severity of RHD may in part be due to the route of infection. Parkes *et al.* (2001; 2002) found that an oral inoculation of virus did not result in serological response in young rabbits, while other studies described seroconversion subsequent to intramuscular inoculation (Robinson *et al.* 2002b). Although direct comparisons were not made in the same experiments, Parkes *et al.* (2001; 2002) postulated that intramuscular infection (and by extension intradermal infection, which is most likely with stinging insects) is more likely to induce immunity than infection *per os*. The authors claimed theoretical support for this hypothesis from reports that ABH blood-group antigens of the epithelial cells of the upper respiratory and digestive tract are necessary to allow RHDV to attach to and penetrate these epithelial cells (Ruvoen-Clouet *et al.* 2000).

Potential arthropod vectors of RHDV in New Zealand

The suspected importance of insects as vectors of RHDV in other countries has drawn attention to the role that arthropods may play in the disease in New Zealand. Crosby and McLennan (1996) classified three species of blowflies, *Lucilia cuprina* (Wiedemann), *Lucilia sericata* (Meigen) and *Calliphora stygia* (Fabricus), as likely indirect or direct vectors of RHDV. In total, eight species of Diptera were identified, including *Hybopygia varia* (Walker) (alternative nomenclature *Oxysarcodexia varia*). This species was demonstrated to be the most likely vector in a study in Otago, New Zealand (Barratt *et al.* 1998). In this field experiment it was shown that caged rabbits exposed to insects in an area where RHD was present, became infected with the disease, and positive RT PCR results were obtained on *H. varia* trapped inside the rabbit cages.

RHDV has been also detected on New Zealand's four most common blowflies: *C. vicina*, *C. stygia*, *C. quadrimaculata* (Svederus) and *L. sericata*, with the last most frequently testing positive for RHDV in Central Otago (Heath *et al.* 1999). RHDV was detectable in higher proportions (71%) in areas of natural introduction of the virus, with

C. quadrimaculata (Svederus) having the highest proportion of positive pools. In contrast, only 36% of pooled fly samples from biocided areas were positive for RHDV, with *L. sericata* being the species with the highest number of positive pools. *L. sericata* and *H. varia* were also the most abundant species trapped in the rabbit prone areas of the South Island. Although no RHDV was detected on these species in another South Island study (Barratt *et al.* 2001), based on previous studies they are still considered as the fly species most likely to be involved in the spread of RHDV.

Although a mite (*Listrophus gibbus*) and a louse (*Haemodipsus venticosus*) are widespread among wild rabbits (Bull 1953), it is considered unlikely that biting insects are vectors of RHDV in New Zealand (Parkes *et al.* 2002).

Arthropod vectors of RHDV in Australia

Experimental studies on haematophagus insects found that European rabbit fleas (*Spilopsyllus cuniculi*), Spanish rabbit fleas (*Xenopsylla cunicularis*) and mosquitos (*Culex annulirostris*), that fed on infected rabbits contained RHDV and were able to infect susceptible rabbits (Lenghaus *et al.* 1994). Cooke (1999a) proposed that fleas may be important for local disease transmission within warrens, while mosquitos are able to carry RHDV over longer distances. He further reported that the mosquito *Ocherotatus (Aedes) postspiraculosus* was able to carry viable RHDV (Cooke 2001). These results are consistent with the analysis of questionnaire data on Australian RHDV releases, which found a strong statistical association (Odds Ratio = 2.7) between high flea infestation and rabbit population decline (Lugton 1999).

The role of other insects, including non-haematophagus species, has been also investigated in Australia, and RHDV was detected in 13 different pools of insect species, including blowflies and bush flies (Westbury 1996). The blowfly, *Calliphora stygia*, was shown to be RHDV positive during the escape of RHDV from Wardang Island (Westbury 1996; Mutze *et al.* 1998), and bushflies (*Musca vetustissima*) were shown experimentally to be capable of transmitting RHDV to susceptible rabbits (Westbury 1996; McColl *et al.* 2002a, 2002b). In another field study, eight different fly species were found to be contaminated with RHDV (Asgari *et al.* 1998). A total of 13 of 16 insect samples (5 fly and 3 mosquito species) collected from several sites in

Australia tested positive for RHDV using PCR, but only one sample contained enough virus to cause infection and death in a susceptible rabbit (McColl *et al.* 2002b).

Factors influencing transmission via insects

Given the strong evidence for the involvement of flies as RHDV vectors, it is implicit that factors that affect fly abundance and activity will influence the transmission of RHDV. In Australia, geographical and seasonal variations in the distribution of fly species led to a great variability in vector populations (Cooke 1999a). Seasonal abundance of vectors, such as *Caenopsylla laptevi iberica* in autumn in Spain, could potentially contribute to seasonal occurrence of RHD outbreaks (Cooke 1999b). Cooke (1999a) commented that RHDV activity is more likely to be linked to the seasonal abundance of fly species collectively, rather than to a specific vector. However, some species have been implicated in certain outbreaks. Fly abundance peaks might coincide with the occurrence of RHD outbreaks, and was probably the mechanism of escape of RHDV from Wardang Island (Wardhaugh and Rochester 1995; Cooke and Fenner 2002; McColl *et al.* 2002b).

Parkes (2000) noted that no published information is available on the seasonal abundance of fly species identified as potential RHDV vectors in New Zealand. However, Cottam *et al.* (1998) has described some annual fly variation in the southern North Island. More recently, Barratt *et al.* (2001) identified the predominant calliphorid and sarcophid species in the most rabbit prone areas of the South Island.

Climatic factors influence fly abundance (Barratt *et al.* 2001; Henzell *et al.* 2002), but the role of particular climate variables on activity peaks of possible RHDV vectors are not fully quantified. Furthermore, the RHDV titre in flies declines over time and successful transmission to rabbits is a function of the distance between rabbits, the distance flies are able to cover, the frequency of feeding by either flies or rabbits, and the decay of the virus. All of these factors are highly likely to be influenced by climatic conditions (Heath *et al.* 1999).

To gain a better understanding of the importance of insect transmission, further knowledge of the probability of viral contamination of insects, and of the period over which RHDV-contaminated insects can infect rabbits is needed (McColl *et al.* 2002b).

Transmission via predators and scavengers

Vertebrate vectors may also play an important role in the spread of the disease. Predators are able to open rabbit carcasses and expose virus to insect vectors and scavengers. Some species that eat rabbits that died from, or were infected with, RHDV can produce antibodies in response to the virus (Simon *et al.* 1994; Leighton *et al.* 1995; Dedek and Frölich 1997; Frölich *et al.* 1998), and could mechanically transmit RHDV to other areas (on their feet or claws). Faeces of experimentally infected dogs were shown also to be a source of virulent virus (Simon *et al.* 1994), suggesting that virus shedding possibly also occurs in some non-target species.

Transport of RHDV on the claws, beaks or in the alimentary tract of rabbit-scavenging birds (e.g. Herring gulls) was described as a possible source of RHDV outbreaks in the UK (Chasey 1994). Likewise, virus transmission by birds was also considered to be responsible for the introduction of RHD to the island of Gotland, 100 km away from the Swedish mainland (Gavier-Widen and Morner 1993).

Transmission by humans

Gregg *et al.* (1991) stated that humans are probably the most important vector for the spread of RHDV between rabbitries and most likely through contaminated hands and footwear. Passive virus transmission through human traffic has been suggested as a possible origin for natural RHD outbreaks in the UK (Chasey 1994) and for dissemination of the disease in Spain (Villafuerte *et al.* 1994). Trade and exhibitions of domestic rabbits (Lölinger *et al.* 1989; Schlüter *et al.* 1990a; Nowotny *et al.* 1992) and exports of rabbit meat (Gregg *et al.* 1991; Morisse *et al.* 1991) have all been attributed as sources of disease outbreaks.

The clinical signs of RHD

Following infection, the incubation period of RHDV is typically 20 to 48 hours, with a maximum of 3 days (Liu *et al.* 1984; Xu and Chen 1989; Patton 1989; Schirrmeyer *et al.* 1990; Marcato *et al.* 1991). In peracute cases, no characteristic clinical signs occur and death happens suddenly after 6-12 hours, with haematuria, vaginal haemorrhage and foamy discharge from the nostrils being observed occasionally (Ohlinger *et al.* 1989; Marcato *et al.* 1991). Several authors describe the peracute form as the most common clinical manifestation of the disease (Liu *et al.* 1984; Xu and Chen 1989; Boujon *et al.* 1989; Schlüter *et al.* 1990b).

In the acute form, affected animals appear quiet with an increased respiratory rate. Clinical signs include anorexia, pyrexia, apathy, dullness, prostration, side recumbency, lacrimation, epistaxis and mucohaemorrhagic nasal discharge, ocular haemorrhaging and cyanoses of mucous membranes, ears and eyelids (Lölinger *et al.* 1989; Xu and Chen 1989; Patton 1989; Nowotny *et al.* 1990; Du 1990; Marcato *et al.* 1991). Death may ensue 12-48 hours after the onset of these clinical signs (Xu and Chen 1989), but sometimes animals die as late as 8 days after infection (Ohlinger *et al.* 1993). Some animals develop severe nervous signs such as convulsions, contractions, ataxia, posterior paralysis, and opisthotonos (Du 1990; Nowotny *et al.* 1990; Schlüter *et al.* 1990b). Body temperature increases in later phases of the infection, with fever up to 41°C (Du 1990), while shortly before death a temperature decrease may occur (Xu and Chen 1989). Relaxation of the anus with constipation or diarrhoea can occur shortly before death, with mucous material or faeces covering the perianal area (Marcato *et al.* 1991). Groans and cries before death are reported (Schlüter *et al.* 1990b; Marcato *et al.* 1991), which are interpreted as pain by some authors (Patton 1989). Cases in pregnant does can lead to abortion of dead fetuses (Marcato *et al.* 1991). If animals recover from the acute initial phase of the disease, severe icteric discolouration of the mucus membranes can persist, with death occurring some weeks later (Marcato *et al.* 1991).

Subacute or mild forms of clinical RHD have been described in the later stages of epidemics (Patton 1989; Xu and Chen 1989; Marcato *et al.* 1991). Animals show more subtle signs and survive (Lölinger 1990; Marcato *et al.* 1991). They also develop antibodies against RHDV that are protective against reinfection (Patton 1989); these

antibody titres gradually decline over time, but can be boosted after re-exposure to RHDV (Cooke *et al.* 2000). Clinical symptoms are present for two to three days (Xu and Chen 1989). Marcato *et al.* (1991) reported a chronic form of RHDV infection that was asymptomatic, but considered to be extremely rare. No more details are given about this clinical form in the literature. A New Zealand study revealed an interesting clinical sign in rabbits shot on several farms (Clark *et al.* 1999). An estimated 1-5% of rabbits showed firm nodular or ridge-like lesions or necrosis in the auricular cartilage resulting in folding of the ear or complete loss of the outer pinna. These findings were significantly associated with higher RHDV antibody titres.

Infection occurs in rabbits of all age groups, but clinical disease is only present in animals older than 5-7 weeks (Goodly 2001). Young rabbits born from antibody-free dams are resistant for up to four weeks while susceptibility increases rapidly thereafter (Mocsari 1990; Smid *et al.* 1991; Morisse *et al.* 1991; Nowotny *et al.* 1993). Using PCR techniques, RHDV RNA was detectable between 1 and 2 days post infection in most organs (and also in faeces) of 4-5 week old rabbits, but persisted only in spleen and bile by the end of the full observation period of 47 days (Shien *et al.* 2000). In addition, antibody titres also persisted for the full 47 days (Shien *et al.* 2000), and it has been suggested that immunity following infection may be lifelong (Cooke and Fenner 2002; Robinson *et al.* 2002b).

Several researchers investigated the pathogenesis of this age-related resistance. The activity of liver enzymes (transaminases) increases with age, and such age-related changes in liver metabolism may be essential for supporting the replication of the virus (Morisse *et al.* 1991). Presence of the VP60 antigen could not be detected in liver tissues of rabbits infected experimentally at younger than four weeks, but was present in very low proportions (0.01-0.2%) in the hepatocytes of six-week old rabbits. It has been inferred that changes in liver structure as rabbits mature in this age range (from six weeks and older) may support viral replication (Prieto *et al.* 2000). Greater susceptibility to RHDV infection of hepatocytes from four week old rabbits compared with those from two-week-old rabbits was also shown histologically (Mikami *et al.* 1999). The authors suggested that dietary changes from predominantly milk to grass diets induce changes in liver function. Another possible mechanism of natural resistance of immature rabbits was demonstrated by Ruvoen-Clouet *et al.* (2000). Binding of

RHDV to antigens of tracheal epithelial cells was weak in 6-week-old rabbits compared to adult rabbits. Furthermore, compared to adult rabbits, the epithelial tissues of young rabbits express very little of the A and H major histo-blood group antigens that are considered important for viral attachment and replication (Ruvoen-Clouet *et al.* 2000).

Apart from this apparently innate RHDV resistance of young rabbits, passive immunity can be passed transplacentally to foetuses from dams that survived RHDV infection. In a field study in Australia, Cooke *et al.* (2000) found that maternal antibodies persisted for up to 11 weeks from birth, with a mean persistence of detectable antibody of about 8 weeks. Experimental studies have shown that the susceptibility to RHDV of kittens born to seropositive does depends on the does' titres rather than the antibody titres of the offspring (Robinson *et al.* 2002b). Kittens can be protected by maternal antibodies up to 13 weeks of age, well beyond the age (5-6 weeks) at which innate resistance has been ascribed to.

The pathology of RHD

Sudden death from RHD results from multiple organ failure including lung oedema, adrenocortical necrosis, circulatory disorders of the kidneys and liver necrosis (Marcato *et al.* 1991). Severe disseminated intravascular coagulation (DIC) leads to acute thrombosis in major blood vessels and capillaries, which in turn results in infarction in many organs (Xu and Chen 1989; Gregg *et al.* 1991). Poor blood coagulation and multifocal haemorrhages occur due to exhaustion of procoagulant factors (Marcato *et al.* 1991). In addition, massive hepatic necrosis, resulting from direct virus replication, is central to RHD pathogenesis, as it initiates DIC which in turn exacerbates the hepatic injury (Plassiart *et al.* 1992). The pathological aspects of RHD, modified from Danner (1995), are summarised in Table 1.

Table 1: Gross findings and microscopic lesions found in RHD infected rabbits

Organ	Gross findings	Microscopic lesions
Liver	- Reduced consistency, in some cases moderately swollen (Boujon <i>et al.</i> 1989; Albert and Wenzel 1990) - Pale yellow or dark reddish colour (Ohlinger <i>et al.</i> 1989; Marcato <i>et al.</i>	- Multifocal necrosis, particularly in the peripheral areas of the lobules, considered to be pathognomonic for RHD (Marcato <i>et al.</i> 1991; Fuchs and Weissenbock 1992) - Necrotic hepatocytes with strong

	1991) • Marked lobular pattern (Ohlinger <i>et al.</i> 1989; Marcato <i>et al.</i> 1991; Zimmer <i>et al.</i> 1992) • Fine granular surface sometimes (Marcato <i>et al.</i> 1991)	homogeneous eosinophilia, partly dystrophic calcification and fibrosis (Boujon <i>et al.</i> 1989; Marcato <i>et al.</i> 1991; Fuchs and Weissenbock 1992) • Early leucocytic exudation in case of acute necrotic hepatitis, with granulocytes and lymphocytes in portal spaces and sinusoids (Marcato <i>et al.</i> 1991; Plassiart <i>et al.</i> 1992; Park <i>et al.</i> 1995) • Anisomorphism in hepatocytes: karyolysis, pyknosis, bile pigment and/or iron pigment deposition, apoptosis, cytoplasmic swelling (Marcato <i>et al.</i> 1991; Fuchs and Weissenbock 1992) • Microvascular steatosis (Marcato <i>et al.</i> 1991; Fuchs and Weissenbock 1992) • Signs of liver cirrhosis in subacute disease form (Teifke <i>et al.</i> 2002)
Spleen	• Splenomegaly (Ohlinger <i>et al.</i> 1989; Marcato <i>et al.</i> 1991) • Dark reddish discolouration (Ohlinger <i>et al.</i> 1989)	• Red pulp hyaline necrosis (Gregg <i>et al.</i> 1991) • Follicular karyorrhexis and lymphopenia (Nowotny <i>et al.</i> 1990; Marcato <i>et al.</i> 1991; Ohlinger <i>et al.</i> 1993) • Follicle arteries with hyaline microthrombi (Zimmer <i>et al.</i> 1992)
Kidney	• Swollen, dull pale (Marcato <i>et al.</i> 1991) to patchy reddish discolouration (Albert and Wenzel 1990; Ohlinger <i>et al.</i> 1993)	• Hyperaemia and haemorrhages in the renal medulla (Marcato <i>et al.</i> 1991; Ohlinger <i>et al.</i> 1993) • Hyaline microthrombi in the glomerular capillaries (Zimmer <i>et al.</i> 1992; Ohlinger <i>et al.</i> 1993) • Tubules with necrotic epithelial cells (Fuchs and Weissenbock 1992; Zimmer <i>et al.</i> 1992)
Lung	• Lung congestion and lung oedema (Schlüter <i>et al.</i> 1990b; Erber <i>et al.</i> 1991; Nowotny <i>et al.</i> 1993) • Petechial and echymotic multifocal haemorrhages (Lölinger <i>et al.</i> 1989;	• Intra-alveolar haemorrhages (Boujon <i>et al.</i> 1989; Fuchs and Weissenbock 1992; Zimmer <i>et al.</i> 1992) • Microthrombi in alveolar capillaries (Nowotny <i>et al.</i> 1990; Plassiart <i>et al.</i> 1992;

	Boujon <i>et al.</i> 1989; Zimmer <i>et al.</i> 1992)	Zimmer <i>et al.</i> 1992) Alveolar oedema (Zimmer <i>et al.</i> 1992; Ohlinger <i>et al.</i> 1993)
Trachea	Hyperaemic, dark reddening of the tracheal mucosa, containing frothy fluid (Ohlinger <i>et al.</i> 1989; Albert and Wenzel 1990; Marcato <i>et al.</i> 1991)	- Hyperaemia of the tracheal mucosa (Marcato <i>et al.</i> 1991; Zimmer <i>et al.</i> 1992) - Leukocyte infiltration (Marcato <i>et al.</i> 1991; Ohlinger <i>et al.</i> 1993) - Calcification of tracheal cartilage (Marcato <i>et al.</i> 1991)
Heart	Often haemorrhages in the myocardium (Marcato <i>et al.</i> 1991; Zimmer <i>et al.</i> 1992)	Subepicardial and myocardial haemorrhages and degeneration of some heart muscle cells (Zimmer <i>et al.</i> 1992)
Gastro-intestinal tract	- Catarrhal gastritis with mucosal erosions (Marcato <i>et al.</i> 1991) - Stomach and caecum obstipation (Boujon <i>et al.</i> 1989) - Sometimes subserous bleedings (Albert and Wenzel 1990)	Necrosis of the small intestinal crypts and subsequent villous atrophy (Gregg <i>et al.</i>, 1991)
Thymus	Often haemorrhages (Marcato <i>et al.</i> 1991; Zimmer <i>et al.</i> 1992)	Necrosis of lymphocytes (Plassiart <i>et al.</i> 1992)
Lymph nodes	- Enlargement of the mesenteric lymph nodes (Marcato <i>et al.</i> 1991) - Occasionally hyperaemia (Boujon <i>et al.</i> 1989)	Karyorrhexis and depletion of lymphocytes (Xu and Chen 1989)
Central nervous system	Occasionally bleeding in spinal cord and brain (Nowotny <i>et al.</i> 1990)	- Intramyelinic oedema and microthrombi (Marcato <i>et al.</i> 1991) - Non-purulent leptomeningitis (Nowotny <i>et al.</i> 1990; Fuchs and Weissenbock 1992)
Blood	Poor blood coagulation (Ohlinger <i>et al.</i> 1989; Marcato <i>et al.</i> 1991)	

Laboratory diagnosis of RHD

Although some caliciviruses can be readily cultured *in vitro*, other members of this family cannot be cultured by conventional virological methods (Xu and Chen 1989; Erber *et al.* 1991; Gregg *et al.* 1991; Park *et al.* 1991). Therefore, while conventional

methods of antibody or antigen detection can be used to diagnose RHDV, bioassays using susceptible rabbits is the only method available to confirm the presence of viable and infective RHDV. Laboratory diagnostic methods can be categorised into methods for detection of virus or viral components, and methods for detection of immune responses of rabbits (Meers 1998).

Detection of RHDV

Electron microscopy (EM) of homogenised rabbit tissues can be used to demonstrate the presence of virus particles that are morphologically consistent with RHDV. Rodak *et al.* (1991) used EM as a screening technique for large numbers of diagnostic samples, and it also has had an important role in characterising RHDV morphology (Smid *et al.* 1989; Valicek *et al.* 1990; Park and Itakura 1992; Alexandrov *et al.* 1993; Zheng *et al.* 2001).

The first test developed for detecting RHDV was the haemagglutination test (Pu *et al.* 1985), which is based on the ability of RHDV to agglutinate human erythrocytes (Liu *et al.* 1984). This test was widely used by Chinese and European scientists as a screening method (Xu and Chen 1989; Nowotny *et al.* 1990; Villafuerte *et al.* 1994; Marchandea *et al.* 1998; Calvete *et al.* 2002). However, the sensitivity and specificity of this method appear to be unsatisfactory. Investigations estimated the specificity of the test to be 92%, and its sensitivity to be in the order of 80 to 90% (Capucci *et al.* 1991; Zimmer *et al.* 1992; Haas and Thiel 1993).

Immunohistochemical diagnosis using immunoperoxidase (Rodak *et al.* 1990b; Carrasco *et al.* 1991; Alexandrov *et al.* 1992; Stoerckle-Berger *et al.* 1992; von Elling 1992) and immunofluorescence techniques (Nowotny *et al.* 1990; Valicek *et al.* 1992; Collins *et al.* 1995; Nawwar *et al.* 1996) have been also applied to detect RHDV antigens. These methods offer the advantage of better sensitivity to detect low levels of virus particles in tissue sections. However, Haas and Thiel (1993) counselled against using immunofluorescence in routine diagnosis, due to the problem of non-specific binding of fluorescent conjugates in uninfected samples. An alternative approach for antigen detection is the use of antigen-capture ELISAs employing monoclonal (Rodak *et al.* 1990b; Capucci *et al.* 1991, 1995; Collins *et al.* 1996) or polyclonal antibodies

(Nardelli *et al.* 1996). Although the latter have been shown to cross-react with liver components, antigen ELISAs employing monoclonal antibodies to RHDV antigens are often used for confirmation of RHDV infection in rabbit tissues.

The most sensitive and specific method for detection of RHDV is the use of the reverse transcriptase polymerase chain reaction (RT-PCR) to detect viral RNA. Lenghaus *et al.* (1994) found liver samples of rabbits tested positive with RT-PCR at dilutions up to 10^{-10} . RT-PCR also has been used successfully to detect viral RNA in rabbit serum (Moss *et al.* 2002) and in tissues of non-target species, including insects (Asgari *et al.* 1998). RT-PCR has the advantage that it can be applied to detect the viral genome in very young rabbits, in which other tests have limitations (Gould *et al.* 1997). By sequencing RT-PCR products, phylogenetic relationships between strains can be made (Nowotny *et al.* 1997; Moss *et al.* 2002; Le Gall-Recule *et al.* 2003) and the evolution of the virus and the genetic drifts determined (Asgari *et al.* 1999). However, Gould *et al.* (1997) recommended the use of RT-PCR in conjunction with other tests, as false positive results due to contamination can occur.

Detection of RHDV antibodies

Immune responses to RHDV comprise both cell-mediated and humoral responses (Meers 1998). Deng *et al.* (1991), cited in Huang (1991), used a monolayer liquid-phase haemagglutination technique to identify antibody-forming and antibody-secreting cells following immunisation against RHD. While this method can provide important information about anti-viral immunity, it is complex and not often used in RHD diagnostics. No other studies on cell-mediated immunity to RHDV have been reported, and available diagnostic techniques are limited to detection of humoral antibody responses.

The haemagglutination inhibition test (HI) was the first test reported to detect RHDV antibodies (Pu *et al.* 1985). HI-tests are in common use and are an inexpensive and simple method for RHDV antibody detection (Berninger and House 1995; Trout *et al.* 1997a; Frölich *et al.* 1998; Elghaffar *et al.* 2000; Mizoguchi *et al.* 2003). However, to avoid false positive results it is necessary to remove natural agglutinins in the serum, and a range of procedures for serum pre-treatment have been used (Maess *et al.* 1989;

Kiepker 1990; Park *et al.* 1991; Collins *et al.* 1995). False positive test results for RHDV were also observed in animals infected with *Pasteurella* spp. (Rodak *et al.* 1991). Another disadvantage of the test is its inability to detect low concentrations of antibodies, which has been observed by some researchers (Danner 1995; Dedek and Frölich 1997).

Indirect Enzyme Linked Immunosorbent Assays (ELISA), for the detection of RHDV antibodies have been developed in Italy (Frescura *et al.* 1989b), Germany (Schirrmeyer *et al.* 1990), Czechoslovakia (Rodak *et al.* 1990a), Australia (Collins *et al.* 1995) and England (Chasey *et al.* 1995). Indirect ELISAs, in common with all serological assays, are often confronted with the problem, that in addition to antigen-specific antibodies, non-specific antibodies may bind resulting in false positive reactions (Capucci *et al.* 1991).

Competition ELISAs for RHDV have been developed by several researchers (Haas and Ohlinger 1990; Capucci *et al.* 1995; Collins *et al.* 1995; Fitzner and Niedbalski 1996). The Capucci competition ELISA (Lavazza and Capucci 1996) is an Office International des Epizooties accepted method for RHDV antibody detection. The competition step is initiated when diluted test serum and RHDV antigen are added to the monoclonal or polyclonal antiserum (Capucci *et al.* 1996a) which coats the solid phase. Virus bound to the solid phase is then labelled with a dilution of enzyme-conjugated monoclonal antibody. After the enzymatic reaction has taken place, the presence of antibodies in the sera is detected photometrically. The titre of a positive test serum is taken as the dilution, which generates an optical density (OD) value of less than 50% of the negative reference serum (at the 1:10 dilution) (Capucci *et al.* 1997; Cooke *et al.* 2000; Zheng *et al.* 2002).

Researchers have used various cut-offs for the competition ELISA to identify a sample as seropositive. Zheng *et al.* (2002) listed serum dilutions as positive, if the serum dilution exhibited 50% inhibition compared with the OD of the negative control serum at a 1:10 dilution, while Cooke *et al.* (2000) only considered samples to be seropositive, if the OD value of the first dilution of the sample was lower than 0.75 times the OD value of the first dilution of the negative reference serum. Similarly Robinson *et al.* (2002a) recommended the use of 75% inhibition as a cut-off for the

detection of truly RHDV positive sera in the presence of cross-reactive antibodies. McPhee *et al.* (2002) demonstrated that test specificity increased to a maximum when a cut-off of 50% at a 1:10 dilution was selected. However the test sensitivity decreased to 39%, and this incorrectly classified a large proportion of samples as seronegative. In addition, the interpretation of the competition ELISA may differ between regions, which makes it difficult to choose a universal cut-off value. Furthermore, age (based on eye lens weight) also has been shown to affect the competition ELISA results (McPhee *et al.* 2002). To avoid any false positive results due to 'background noise' of the ELISA procedure, Parkes *et al.* (2002) only considered a sample to be positive at the higher dilution of 1:40 (when the OD of the sample inhibits the value of the negative control at 1:10 dilution by more than 50%). In general, Australian studies on RHDV in wild rabbit populations consider serum samples positive at a dilution of 1:10 (Cooke *et al.* 2000; Mutze *et al.* 2002), while New Zealand researchers primarily use the 1:40 dilution (Reddiex *et al.* 2002; Parkes *et al.* 2002). Therefore, serological results from different sources should be interpreted with caution.

It has become generally accepted that the competition ELISA is more specific than the indirect ELISA (Capucci *et al.* 1991). This was well demonstrated in a study of Robinson *et al.* (2002a) where a significant proportion of positive cross-reactors were detected in Australian rabbit sera only with the indirect ELISA, but not with the competition ELISA. Because the Capucci competition ELISA fails to distinguish between pathogenic RHDV and cross-reactive RHDV-like virus it is only adequate for the detection of RHDV in a rabbit population, and should be used with caution for estimating prevalence of RHDV immunity (McPhee *et al.* 2002).

RHDV specific antibodies (IgG, IgA and IgM) can be determined by using isotype ELISA (Capucci *et al.* 1997) to distinguish between different classes of immunity (maternal antibodies, recent infection and past infection, re-exposure to RHDV and an indication for exposure to RHDV-like agent) (Cooke *et al.* 2000), to compare vaccinated and naturally exposed rabbits (Capucci *et al.* 1997) and to specify the infection process in single animals in more detail (Cooke *et al.* 2002). These tests are an important tool to clarify test results based on the competition ELISA. Procedures differ between isotype classes. In IgA and IgM ELISAs, diluted rabbit serum is added to wells coated with anti-IgM or anti-IgA monoclonal antibodies, followed by the addition of

RHDV antigen and enzyme-conjugated anti-RHDV monoclonal antibodies. In IgG ELISAs, plates are coated with anti-RHDV monoclonal antibodies, followed by the addition of RHDV, test serum and enzyme-conjugated anti-IgG monoclonal antibodies. After washing the plates, substrate is added and the absorbance measured. The titre of a serum is expressed relative to the positive reference serum (Cooke *et al.* 2000) or to the serum control at 1:40 dilution without RHDV (Zheng *et al.* 2002).

Epidemiology of RHD in wild rabbits, with emphasis on New Zealand

Europe

Most European reports on the initial impact of RHD focus on domestic rabbit populations, and detailed studies on wild rabbits have been limited to Spain and France. In these countries, rabbits are significant game species and also play an important role in ecological systems. In general, following the emergence of RHD, wild rabbit numbers declined in all European countries, but the decreases were relatively small in Britain and Scandinavia compared to other countries (Cooke and Fenner 2002).

From 1988 to 1990, the rabbit mortality in initial RHD outbreaks in Spain was estimated to be between 55% to 75% (Villafuerte *et al.* 1994, 1995). Interviews with hunters indicated that mortality risks declined to around 30% in outbreaks in subsequent years, with annual outbreaks concentrated in the spring and winter seasons (53% and 34% of outbreaks respectively). Recovery of rabbit numbers was slow in many sites with low population densities, but more rapid in dense populations. It has been suggested that high population densities facilitate the transmission of RHD and increase mortality, resulting in a reduction in the median age of the population to the point where rabbits are no longer susceptible when challenged (Calvete and Estrada 2000, cited in Cooke and Fenner 2002). These surviving animals would be recruited into the new breeding population, leading to reduction in the overall RHD mortality.

Similar to the observations in Spain, RHD spread slowly throughout France and did not reach some areas until several years after its first occurrence in 1998 (Marchandeau *et al.* 1998). Crude mortality estimates of up to 88% were recorded in the first year of

RHD occurrence, with mortality directly attributed to RHD estimated to be approximately 45%. A wild rabbit population studied near Paris did not recover within three years from its initial epidemic, after which time only one more outbreak occurred despite serological evidence of ongoing presence of the virus (Marchandeau *et al.* 2000).

Australia

The initial spread of RHD through Australia appeared to be influenced by climatic and geographical patterns. In arid and semi-arid regions population declines between 60 to 97% were observed (Bowen and Read 1998; Mutze *et al.* 1998; Cooke 1999a), while in more moist areas in New South Wales the initial impact of the disease was variable. Reduction in rabbit numbers of between 68-91% occurred in populations in some moist areas, while substantial increases of up to 87% above pre-RHD density were observed in others (Saunders *et al.* 1999). In South Australia, rabbit numbers were reduced in the longer term by 85% in arid areas, but only by 12% in coastal areas (Mutze *et al.* 2002). In addition, seasonal peaks in rabbit abundance shifted from spring to late summer (Mutze *et al.* 2002).

New Zealand

Rabbit numbers are generally lower in the North Island of New Zealand than in the South Island. On 23 farms where RHDV was released between late 1997 and early 1998, rabbit density was estimated to be between 2 and 37 rabbits per spotlight kilometre (median 13) (Sanson *et al.* 2000). The estimated reduction of rabbit numbers was in the order of 50%, but highly variable (0% to 77%). Although 80% of surveyed farmers reported that the disease spread from the release locations, only 27% of these farmers indicated that dead rabbits were found more than 300 m away from the release sites.

A serological survey from several sites in Central Otago in the South Island of New Zealand was conducted on sites where RHDV had been deliberately released and subsequently spread naturally (O'Keefe *et al.* 1999). Higher survival rates and levels of antibodies were observed at sites where RHDV was spread on carrots or oats than on

properties to which RHD had spread naturally. It has been suggested that large-scale baiting could result in exposure of rabbits to inactivated RHDV, which in turn could induce protective immunity. Further investigations of RHDV in the South Island indicated population reduction of more than 90% in high rabbit density areas (39-69 rabbits per spotlight kilometre pre-RHD). Epidemics recurred annually and apparent seroprevalence in survivors was approximately 35% at both biocided sites and sites where the virus spread naturally (Parkes *et al.* 2002). In areas of the Mackenzie Basin with lower rabbit densities, population reductions were in the order of 50%, epidemics recurred biannually and seroprevalence in survivors was approximately 20%. In contrast, some areas in North Canterbury experienced minimal population reductions and 50-80% of surviving rabbits were seropositive. The existence of another RHD-like calicivirus that might protect against RHD in New Zealand has been postulated, but remains unproven (Zheng *et al.* 2002). Some ecological concerns exist that a rapid reduction in rabbit numbers could lead to increased short-term predation on other non-pest species in New Zealand, and in particular native birds and their eggs (Haselmayer and Jamieson 2001; Norbury *et al.* 2002). However, it has been put forward that decreases in rabbit predator numbers (feral cats and ferrets) following declines in rabbit populations may yet yield long-term benefits for native fauna (Norbury *et al.* 2002).

General patterns of disease occurrence

The rate of local spread of RHD during the initial epidemic in Central Otago was estimated to be about 200 m per day (or 6 km per month) with the prevailing wind (Parkes *et al.* 1999). This was considerably less than estimates made in Spain (2 to 10 km per month) (Villafuerte *et al.* 1995) and Australia (9 to 414 km per month) (Kovaliski 1998), but greater than a figure of less than one kilometre per month reported for England (Trout *et al.* 1997b). Vectors are one factor that could affect the rate of spread of RHDV. The likely variability in vector abundance between seasons will be influenced by climatic factors that may also affect the survival of the virus and the timing and duration of rabbit breeding. Seasonal peaks of RHD epidemics are expected to occur in association with increased rabbit production, especially after young animals have lost their maternal immunity (Cooke and Fenner 2002).

In Spain, RHD outbreaks typically lasted four to five weeks and occurred during the main breeding season from late autumn to early spring, but predominantly during the winter months (Cooke 2002; Calvete *et al.* 2002). Deaths of seronegative does most likely contributed to the death of suckling offspring. In addition, outbreaks late in the breeding season resulted in a 50% mortality of juvenile animals, probably as a direct result of them no longer being protected by innate age-related resistance or maternally-derived passive immunity (Cooke 2002). Maximum mortality in adults was also observed in the second half of the breeding season, when the highest proportion of females were found (Calvete *et al.* 2002).

In semi-arid inland areas of Australia, RHD epidemics were also observed in the beginning of the breeding season in late autumn-early winter (May-July). These epidemics killed up to 87% of young adults and was probably directly attributed to a loss of maternal immunity over the summer months (Mutze *et al.* 2002; Cooke and Fenner 2002). The observation that animals born late in the same season were usually not affected was attributed to the possibility that RHDV did not persist (outbreaks rarely occur in summer) until the time when these late-born animals became susceptible. These animals were then incorporated into the new breeding population, and are susceptible to infection when cooler temperatures predispose to RHD outbreaks the following autumn.

RHD outbreaks in dry inland areas of Central Otago in the South Island also were reported in the autumn months. Affected carcasses were found up to 40 days after the first case was reported on sites with naturally introduced outbreaks, and up to 80 days on biocided sites (Parkes *et al.* 2002). In contrast, RHD outbreaks occurred in late spring to early summer in cooler regions of North Canterbury, and post-emergent mortality was highest in early-born rabbits (August-October) as they became susceptible to RHD in the late breeding season (Reddiex *et al.* 2002). Prior to the introduction of RHDV to New Zealand, modelling of the likely spread of RHD predicted that outbreaks would occur in summer or autumn. In these relatively simple models, all seasonal factors other than rabbit production (such as virus-half life) were assumed constant, although some of these factors are probably important determinants for the timing of outbreaks (Barlow and Kean 1998). Subsequent modelling work predicted yearly epidemics alternating between spring and winter (Barlow *et al.* 2002). However, despite juvenile recruitment that supported post-epidemic population

recovery over summer, no second outbreak was observed on field sites in Otago. The authors suggested that a low recruitment in the spring-summer period following the RHD release, for instance due to predation or poor environmental conditions, could result in rabbit density failing to reach a threshold level required to sustain a new epidemic in the following winter (Barlow *et al.* 2002).

Several authors have observed a relationship between RHD and other causes of rabbit mortality. The interaction between mortality due to RHD and patterns of predation in juvenile rabbits was investigated in a study in North Canterbury, New Zealand (Reddiex *et al.* 2002). In this study, it was observed that population declines were significantly lower at sites where predators had been removed compared to sites where predator populations were not removed. In a Spanish study, predation continued to be the predominant cause of rabbit mortality, accounting for 60% of rabbit deaths (Calvete *et al.* 2002). Increased rabbit numbers due to a decrease in predation was thought to increase the basic reproduction rate of RHDV and hence RHD deaths, which partly compensated for the decrease in predation. In France, predation by red foxes may have delayed population recovery after an initial RHD epidemic, and then restricted rabbit numbers to levels at which further RHD was undetected (Marchandeau *et al.* 2000).

Furthermore, interaction between myxomatosis and RHD has been described. Immunosuppression by the *Myxoma virus* could contribute to higher mortalities in rabbits exposed to RHD, despite these animals having high RHDV antibody titres (Marchandeau *et al.* 1998). The timing of myxomatosis epidemics in Australia may be affected by RHD outbreaks, and be delayed from late spring to early summer and autumn (Mutze *et al.* 2002).

A frequent impression, that has not been thoroughly investigated in field studies, is the apparent patchiness of the disease. Several attempts were made to model the disease, which appears to exhibit considerable spatial heterogeneity. Barlow and Kean (1998) and Barlow (1999) postulated a hide and seek phenomenon for RHD in which recurrent epidemics at a location could result in reintroduction of the agent. In this scenario, the agent would not persist at an outbreak site following an epidemic, but would be dispersed to surrounding sites by dispersal or vectors. Reintroduction from other

infected sites could then be repeated when the population had recovered. This phenomenon depends on the existence of spatial heterogeneity, the scale of which would be affected by the mobility of vectors and the rapidity with which the disease occurred. However, this cannot explain the epidemic pattern in which many, small, partially synchronised, epidemics occur.

Variability in rabbit population demography as described by Trout *et al.* (2000) could result in different levels of risk for neighbouring populations (White *et al.* 2001). One modelling study explored the scenario in which pathogenic and non-pathogenic strains occurred, and concluded that the strain with the higher basic reproduction rate would dominate (White *et al.* 2001). The authors suggested a spatial effect on population immunity, where neighbouring populations that are protected by a non-pathogenic strain of the virus could shield an area of high RHDV mortality risk (White *et al.* 2001). This model was later modified by using chronic infection and acute infection states to explain spatial heterogeneity, rather than the presence of a non-pathogenic virus (White *et al.* 2002). An individual-based spatial model to describe RHD in European wild rabbit populations was developed recently, but was mainly used to describe the current situation, rather than to provide new insights into the disease process (Fa *et al.* 2001). Unfortunately, quality ecological models of RHD epidemiology are restrained by a marked lack of longitudinal seroprevalence data before and after epidemics, and a lack of accurate data recording the relationships of epidemics with population dynamics (White *et al.* 2001).

Omissions in the literature

This review of the literature on RHD has brought several shortcomings to light. Although the aetiological, clinical and pathological aspects of RHD are well documented, there is a clear deficit of longitudinal studies on the disease in wild rabbit populations. Modellers of RHD epidemiology consistently point out the lack of sufficient information about RHD to validate their models. In addition, RHD is only one source of mortality in wild rabbits, and its likely interrelationships with other causes of mortality require more intensive investigation.

Secondly, although the temporal patterns of outbreaks of RHD often have been described, there has been little attention given to spatial aspects of the disease, although

the variable severity and impact of the disease on rabbit populations ('patchiness') is commented on in several studies. There has been no study that has investigated the spatial occurrence of RHD in relation to rabbit activity, in particular on the home ranges of the animals.

Thirdly, rabbits play an important part in the ecology of other species, most notably rabbit predators and scavengers. Seroconversions in these species have been reported, but were never related to temporal aspects of RHD outbreaks. To understand RHDV behaviour in non-target species, predators and scavengers have to be monitored in a longitudinal study.

Although potential arthropod vectors for RHDV have been described, there is lack of detailed information on fly abundance in relation to RHD outbreaks. The likely importance of climate on the occurrence of RHD outbreaks has been noted, but detailed analyses of climatic factors that influence the abundance of potential vector species are not available in the literature.

Furthermore, there is a paucity of data on the properties of RHDV with respect to its persistence in the environment, most probably because the virus is not culturable. Knowledge of the survival of RHDV in the environment is important for understanding the epidemiology of the disease, and this question warrants further investigation. In New Zealand, the use of RHDV contaminated baits has become a common practice for rabbit control. However, there are concerns that the efficiency of this approach will be undermined if inactivated RHDV on baits in the field can induce protective immunity in rabbits. This hypothesis has yet to be directly tested.

Finally, initial research on RHDV use in New Zealand has mainly focused on RHDV outbreaks, and neglected the use of other control methods that are currently employed. Rabbit control has been practised for more than a century in New Zealand, yet RHDV is a very new disease. Its patterns of use by farmers and their perceptions about its performance have not been formally assessed. Nevertheless, New Zealand farmers introduced RHDV and their awareness of its use and its benefits has to be investigated.

The present research project: outline of research aims and intended outcomes

In this chapter, I have discussed the epidemiology of RHD, which is a relatively new disease in New Zealand. To understand any infectious disease at a population level, it is essential to determine the biological characteristics of the infectious agent and its behaviour in the host, methods for the identification of the agent and of host responses to infection, and the nature of disease transmission. The purpose of my research project was to advance understanding of the epidemiology of RHDV in the first four years after its introduction to New Zealand. The natural history of RHD in the lower North Island of New Zealand was described in a longitudinal observational study that included intensive observations on a well characterised population of individually identified rabbits. Multiple aspects of rabbit ecology had to be considered, as RHD is only one of numerous causes of death in rabbits. The temporal and spatial dynamics of RHD in the population were determined, together with the likely involvement of non-target vertebrate species and arthropods as potential disease vectors. Experimental studies were also conducted to obtain a better understanding of the persistence of the virus in the environment. Finally, farmers were surveyed to understand the perceptions of the users and beneficiaries of RHD with respect to the utility of RHD as a tool in rabbit control.

The overall body of work was founded on the belief that better understanding of the natural occurrence of RHD is essential for appropriate and optimal employment of RHDV as a bio-control agent for rabbits in New Zealand.

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Rabbit in modified single capture cage trap

Chapter 2

A three-year study of *Rabbit haemorrhagic disease virus* infection in a wild rabbit population in New Zealand*

*Submitted by J. Henning, D. U. Pfeiffer, P. R. Davies, J. Meers and R. S. Morris to *Wildlife Research*

Abstract

A longitudinal capture-mark-recapture study was conducted to determine the temporal dynamics of rabbit haemorrhagic disease (RHD) in a low to moderate rabbit density population on sand hill country in the lower North Island of New Zealand. A combination of sampling (trapping and radio-tracking) and diagnostic (cELISA, PCR and isotype ELISA) methods was employed to obtain data weekly from May 1998 until June 2001. Although *Rabbit haemorrhagic disease virus* (RHDV) infection was detected in the study population in all three years, disease epidemics were evident only in the late summer or autumn months in 1999 and 2001. Overall, 20% of 385 samples obtained from adult animals older than 11 weeks were seropositive. An RHD outbreak in 1999 contributed to an estimated population decline of 26%. A second RHD epidemic in February 2001 was associated with a population decline of 52% over the subsequent month. Following the outbreaks, the seroprevalence in adult survivors was between 40 and 50%. During 2000, no deaths due to RHDV were confirmed and mortalities were predominantly attributed to predation. Influx of seronegative immigrants was greatest in the 1999 and 2001 breeding seasons, and preceded the RHD epidemics in those years. RHD epidemics require the population immunity level to fall below a threshold where propagation of infection can be maintained through the population, and then the epidemic is initiated and maintained by both direct and indirect transmission mechanisms. Inter-epidemic virus persistence is likely to be in carrier animals.

Introduction

Feral rabbits reached pest proportions in New Zealand in the mid 1940s and pose a major threat to the agricultural productivity of the country (Gibb and Williams 1994). While conventional methods for rabbit control have been employed for decades, both farmers and the general public expressed optimism about the potential for *Rabbit haemorrhagic disease virus* (RHDV) to combat the rabbit problem (Wilkinson and Fitzgerald 1998; Henning *et al.* 2003a). However, legal approval to introduce the virus was denied due to concerns about ecological and epidemiological consequences (O'Hara 1997). When RHDV was illegally released in the South Island of New Zealand in 1997 (Thompson and Clark 1997), the long-term benefits and risks of its establishment were

uncertain. Subsequent to the initial outbreak of rabbit haemorrhagic disease (RHD) near Himatangi in the Manawatu region of the North Island, we conducted a three-year longitudinal study to evaluate RHDV infection and immunity in a low-density wild rabbit population. This publication is the first in a series of three papers describing the spatial and temporal aspects of RHDV in wild rabbits and non-target species.

Materials and Methods

Study site

The study site was located in rabbit-prone sand country near Himatangi (longitude: 175.300, latitude: -40.425) in the southern part of the North Island of New Zealand, Aotearoa. The site was comprised of approximately 10 ha of dense vegetation, consisting of gorse (*Ulex europaeus*), broom (*Cytisus scoparius*) and toi toi grass (*Cortaderia* sp.) that was surrounded by 20 ha of pasture grazed periodically by beef cattle or sheep. In this region most rabbits dwell above the surface as opposed to living in burrows (Henning *et al.* 2003b). Based on spotlight night counts, rabbit density in the area prior to the study was estimated to be 3 to 4 rabbits per square kilometre. The first confirmed RHD case on the study site occurred by natural spread (i.e. in the absence of baiting by the farmer) in March 1998.

Data collection

A capture-mark-recapture and radio-telemetry study was initiated in May 1998 and continued until June 2001. Trapping and sampling was suspended in the months of October to December 2000, but radio-tracking was conducted throughout this period. Trapping was carried out on two nights per week. A trapping technique was devised in which traps were integrated in a rabbit-proof fence that was constructed between rabbit denning areas of dense vegetation and feeding areas of pasture. The fence consisted of wire-mesh netting extending from 20 cm beneath to 120 cm above the ground, and enclosed an area of approximately 2.5 ha. A total of 86 traps of 7 different designs were employed. No bait was used in the traps and rabbits were caught as they passed through the traps.

Trapped animals were anaesthetised by intramuscular injection of a combination of ketamine hydrochloride (100 mg/ml; Phoenix Pharm Distributors Limited, Auckland, New Zealand) and xylazine (20 mg/ml; Phoenix Pharm Distributors Limited, Auckland, New Zealand) to facilitate examination. All rabbits were identified with metal ear tags (893 JIFFY, Size 3, National Band & Tag Co., Newport, Kentucky, USA). At each capture, rabbits were weighed, measured (body, ear length and hind leg length), and scored for body condition (five scores increasing from emaciated to fat). The pregnancy and lactation status of does was determined by palpation (Dunsmore 1974; Gibb *et al.* 1978) and testicles of bucks were measured. A blood sample (0.5 to 1 ml) was collected for serology from *V. auricularis caudalis*, *V. auricularis rostralis*, *A. auricularis caudalis r. intermedi*i or *V. saphena medialis*. In order to locate and retrieve cadavers for necropsy, radio transmitters (Titley Collars, Waipara, New Zealand or Sirtrack collars, Sirtrack Limited, Havelock North, New Zealand) were attached to rabbits for tracking with a portable radio telemetry receiver (Model CE 12, Custom Electronics of Urbana Inc., Urbana, Illinois, USA). Post mortem examinations were performed on all carcasses recovered, and samples collected for histology and virus detection.

Serology

Serum samples were tested for antibody to RHDV using a competition ELISA (Capucci *et al.* 1991) at four dilutions (1:10, 1:40, 1:160 and 1:640). A sample was considered positive when the corresponding optical density (OD) value of the sample at the 1:40 dilution reduced the OD value of the negative control at 1:10 dilution by more than 50% (Parkes *et al.* 2002). The titre of a positive sample was determined as the highest dilution reducing the OD value of the negative reference serum at 1:10 by at least 50% (Zheng *et al.* 2002).

All samples that were positive on competition ELISA were subsequently tested at three dilutions (1:40, 1:160 and 1:640) with isotype ELISAs (Capucci *et al.* 1997) to determine the presence of IgG, IgA and IgM antibodies to RHDV. A 1:40 dilution of each serum sample, in the absence of RHDV antigen, was used as a serum control. A sample was deemed positive if the OD value at 1:40 in the presence of RHDV was more than 0.2 units above its serum control (Zheng *et al.* 2002). The titre for each serum was the highest dilution at which the OD exceeded that of its serum control by at least 0.2 units.

Our classification of rabbits by immune status (Table 2) was adapted from Cooke *et al.* (2000; 2002), Robinson *et al.* (2002a; 2002b), and Parkes *et al.* (2002).

Table 2: Categories of rabbit immune status to RHDV based on results of the competition and isotype ELISAs [adapted from Cooke *et al.* (2000; 2002), Robinson *et al.* (2002a; 2002b), Parkes *et al.* (2002)]

	c-ELISA ^a	IgG	IgA	IgM	Comment
Pre-existing antibodies ^b	$\leq 1:10$	$\geq 1:640$	none	none	
Maternal immunity	$\geq 1:40$	$\geq 1:40$	none	none	≤ 11 weeks
Recent infection	$\geq 1:40$	$\geq 1:40$	$\geq 1:40$	$\geq 1:640$	
Past infection	$\geq 1:40$	$\geq 1:40$	$\geq 1:40$ or none	$\geq 1:40$ or none	
Re-infection	$\geq 1:40$	$\geq 1:40$	$\geq 1:160$	none	

^a The titre of each serum was taken as the dilution reducing optical density by 50% relative to the negative reference serum.

^b Antibodies to the RHDV-like virus.

Age classification

Age estimates were derived from body weight changes over time using the Gompertz growth model (Cowan 1983; Webb 1993; Henning, unpublished data). Rabbits were categorised into two age groups of less than/equal to, or older than, 11 weeks (77 days), representing immature and adult rabbits respectively. The selection of 11 weeks for dichotomising age groups was based on two considerations. Firstly, young rabbits lose maternal antibodies between 5 and 11 weeks (Cooke *et al.*, 2000), hence seropositive reactions in rabbits older than 11 weeks should indicate RHDV infection after loss of maternal immunity. Secondly, when we examined the frequency distribution for age at first trapping (Figure 1) it was bimodal, suggesting the existence of two sub-populations of rabbits, with the minimum between modes occurring at approximately 80 days.

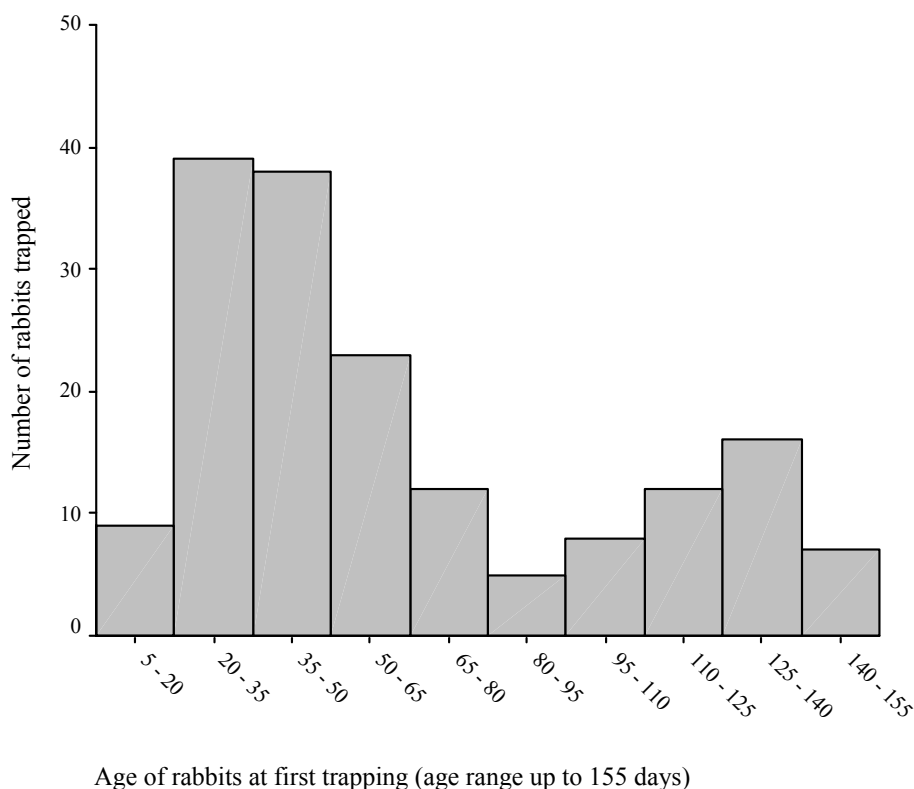


Figure 1: Histogram for age at first capture for animals up to 155 days of age.

Detection of RHDV infection in dead rabbits

Rabbit haemorrhagic disease was confirmed by detection of RHDV by reverse transcriptase polymerase chain reaction (RT-PCR) on liver tissues of rabbit carcasses using methods and primers described by Nowotny *et al.* (1997).

Rabbit abundance

Abundance of rabbits is often measured as number of animals alive, based on repeated trapping data (Henderson 1979). We estimated rabbit abundance from both trapping and radio-tracking data to calculate the number of animals alive. If an animal was trapped on a subsequent trapping occasion or if it was radio-tracked, but not trapped in the same month, it was deemed to be alive. Abundance data were calculated by month and season. The breeding activity of the population was assessed as the proportion of female rabbits older than 4 months that were pregnant, this being the youngest age at which females are found pregnant at autopsy (Fraser 1988).

Monthly (or seasonal) serological and mortality data

For rabbits sampled once in a month (or season), seropositivity was defined by that single test result. Rabbits sampled more than once in a month (or season) were deemed seropositive for that month if any test result was positive. Animals were deemed seronegative for any month in which all test results were negative. Seasons were defined as following: Spring - September, October and November; Summer - December, January and February; Autumn - March, April and May; and Winter - June, July and August.

Prevalence was calculated as the proportion of seropositive animals among all animals examined. Chi-squared and Fisher's Exact tests were used to test for differences between proportions (Dawson-Saunders and Trapp 1994). Monthly mortality incidence density (deaths per 1,000 rabbit-days) was calculated to quantify the rapidity with which new cases (mortality) occurred (Thrusfield 1997) using exact denominators of the sum of days at risk for all animals in the population.

Results

Over a total of 503 days of trapping and radio-tracking, 847 rabbit captures and 1,021 radio-tracking events were recorded, and 817 serum samples were collected.

Rabbit population dynamics

The estimated rabbit population fluctuated over time with peaks observed in the summer months due to recruitment of young rabbits (Figure 2). Peaks in mortality incidence coincided with confirmed cases of RHD in March/April 1999 and February 2001.

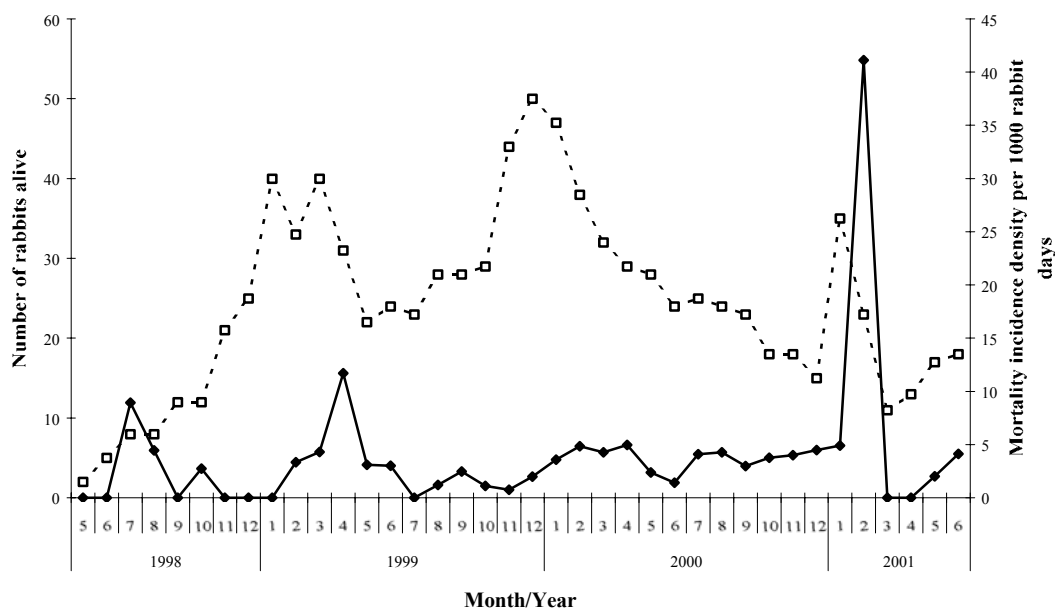


Figure 2: Number of rabbits alive (dashed line) and mortality incidence density per 1000 rabbit days (solid line) for a rabbit population in Himatangi, New Zealand between May 1998 and June 2001.

Pregnant females were present during most months of the study (Figure 3).

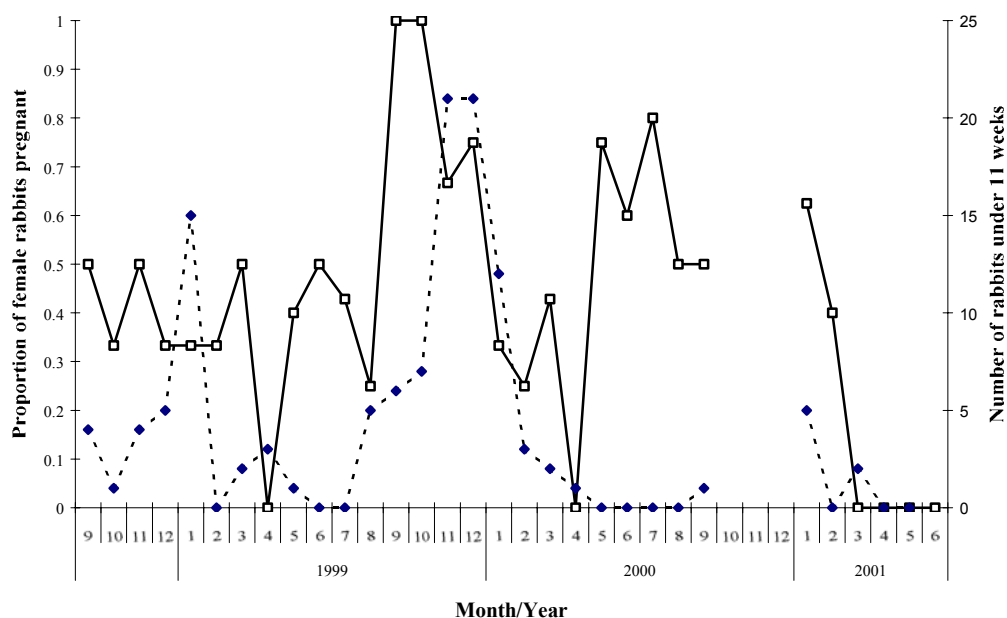


Figure 3: Proportion of female rabbits pregnant (solid line) and numbers of rabbits under 11 weeks (dashed line) in Himatangi, New Zealand between September 1998 and June 2001.

However, peaks and troughs in the prevalence of pregnancy were evident, and no females in palpable stages of gestation were observed during April of any year.

Pregnancy prevalence was highest in the spring months of 1999, when up to 100% of females were detected pregnant. Naturally, the highest peak of young rabbits followed this peak in pregnancy prevalence.

The proportion of immature ($\chi^2 = 88.9$, $df = 12$, $P < 0.0001$) and adult rabbits varied between seasons and year, with rabbits younger than 11 weeks of age being least common during the autumn months (Table 3). The gender distributions of immature rabbits ($\chi^2 = 15.29$, $df = 11$, $P = 0.158$) and adult rabbits ($\chi^2 = 14.36$, $df = 12$, $P = 0.279$) did not vary between seasons - year periods (data not shown).

Table 3: Age distribution [numbers (%)] of rabbits alive (based on trapping and radio-tracking events) by season and year

Season - year	≤11 weeks	>11 weeks	Total
Winter 1998	6 (46%)	7 (54%)	13
Spring 1998	11 (41%)	16 (59%)	27
Summer 1998/1999	20 (43%)	27 (57%)	47
Autumn 1999	4 (9%)	39 (91%)	43
Winter 1999	5 (15%)	28 (85%)	33
Spring 1999	30 (57%)	23 (43%)	53
Summer 1999/2000	37 (59%)	26 (41%)	63
Autumn 2000	4 (11%)	33 (89%)	37
Winter 2000	3 (10%)	27 (90%)	30
Spring 2000 ^a	5 (22%)	18 (78%)	23
Summer 2000/2001 ^b	6 (16%)	32 (84%)	38
Autumn 2001	2 (11%)	16 (89%)	18
Winter 2001 ^c	0 (0%)	18 (100%)	18
Total	133 (30%)	310 (70%)	443

^a No trapping in October and November 2000, ^b no trapping in December 2000, ^c only trapping in June 2001.

Peaks in monthly mortality rate (Figure 2) occurred in July 1998 (9 deaths per 1,000 rabbit-days), in April 1999 (12 per 1,000 rabbit-days) and in February 2001 (42 deaths per 1,000 rabbit-days). The last two peaks were associated with outbreaks of RHD confirmed by detection of viral RNA in carcasses. The outbreak in March/April 1999

contributed to an estimated 26% decline in the study population. The second RHD outbreak in February 2001 was associated with the highest monthly mortality recorded during the study, and the estimated number of rabbits alive decreased by 52% in the following month. Although substantial mortality was observed over several months (particularly February to April) in 2000, there were no confirmed cases of RHD, and deaths were predominantly attributed to predation (Henning *et al.* 2003b). The mortality peak in the winter of 1998 was entirely associated with predation (Henning *et al.* 2003b).

Serology of adult (>11 weeks) rabbits

636 serum samples were collected from animals older than 11 weeks, with up to 35 samples collected from individual animals. After exclusion of multiple samples from individuals within months as described above, 385 results from 128 animals were used for analysis. Seroprevalence increased to 44% after the RHD outbreak in March/April 1999, before declining to 7% by January 2000 with detectable titres persisting in survivors for several months following the epidemic (Figure 4).

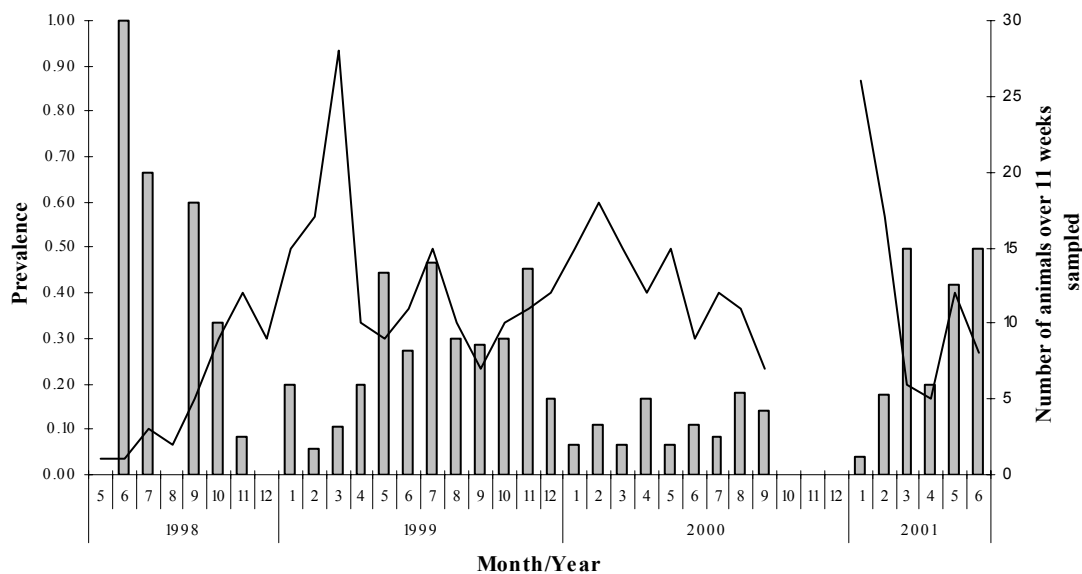


Figure 4: Number of rabbits older than 11 weeks sampled monthly (line) and temporal pattern of seroprevalence of RHDV for this age group (bars) in Himatangi, New Zealand between May 1998 and June 2001.

Similarly, seroprevalence in adult rabbits increased to 50% among survivors of the RHD epidemic in 2001 with individual adult rabbits that were sampled multiple times having antibody titres that remained stable for several weeks. Declining titres were observed in only four (17%) of 23 seropositive animals sampled on several occasions over at least 5 weeks. Although no mortalities attributable to RHDV were observed in the autumn of 2000, during this period there was an apparent increase in seroprevalence to 17%, which persisted for several months. Overall, 77 (20%) of 385 monthly samples obtained from animals older than 11 weeks were seropositive.

The population experienced considerable influx of immigrant animals (newly-trapped animals older than 11 weeks of age), which constituted 36.2% of trapped rabbits across all seasons (Figure 5). To analyse seasonal patterns of immigrant activity, the first trapping season of winter 1998 was excluded and rabbits trapped in this period were deemed to constitute the initial resident population. Trapping was discontinued for the last two months in spring 2000. To prevent immigrants entering the site during this period from being misclassified as immigrating during the summer of 2000/2001, 30% of immigrants amongst rabbits first trapped in summer 2000/2001 were reallocated to the spring months (approximating the expected proportion had spring trapping continued). Adopting this conservative approach, the proportion of immigrant animals varied between seasons ($\chi^2 = 22.3$, $df = 11$, $P = 0.023$), and was highest in spring-summer of 1998/1999 and summer 2000/2001. Males (64%) predominated ($\chi^2 = 6.9$, $df = 1$, $P = 0.012$) among the 84 rabbits classified as immigrants from spring 1998 until winter 2001.

The serological status of newly-trapped animals older than 11 weeks (probable immigrants) and re-trapped animals older than 11 weeks (resident adult population) was compared.

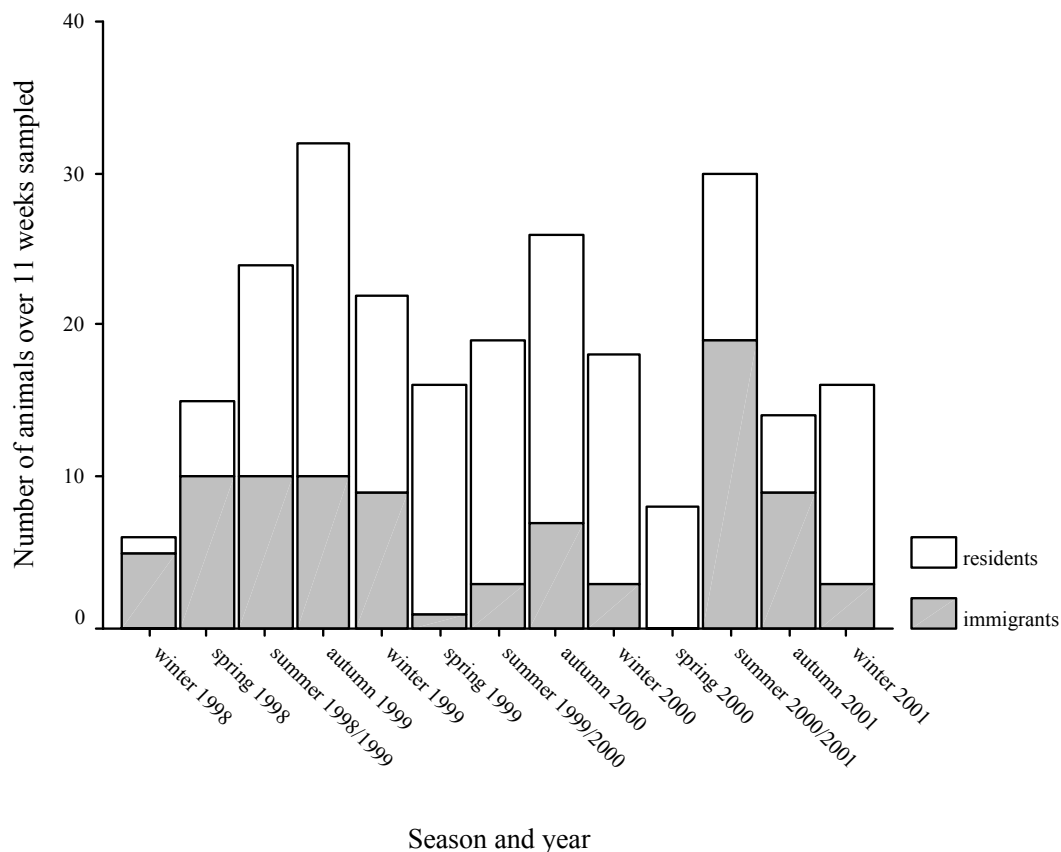


Figure 5: Number of adult animals (> 11 weeks) sampled by season, stratified for residents and immigrants in Himatangi, New Zealand between winter 1998 and winter 2001.

Again excluding data from the initial trapping season in winter 1998, no significant differences in seroprevalence between seasons (Table 4) were observed for adult resident rabbits ($\chi^2 = 13.1$, $df = 11$, $P = 0.259$). Seroprevalence was similar between sexes with 29% of male adult resident rabbits and 25% of female adult resident rabbits being seropositive across all seasons, and the overall prevalence of residents was 26.9%.

Overall the seroprevalence of RHDV in immigrant animals was 16.7%, and fluctuated ($\chi^2 = 14.4$, $df = 11$, $P = 0.099$) between seasons with more immigrants trapped in the autumn-winter period tending to be seropositive, and more immigrants trapped in the spring-summer period tending to be seronegative (Table 4). Seroprevalence was similar between male (19%) and female immigrants (13%).

Table 4: Number of resident and immigrant seropositive and seronegative adult rabbits for RHDV by season

	Immigrant adult rabbits			Residential adult rabbits		
	Negative	Positive	Total	Negative	Positive	Total
Winter 1998 [*]	-	-	-	3 (50%)	3 (50%)	6 (100%)
Spring 1998	9 (60%)	1 (7%)	10 (67%)	2 (13%)	3 (20%)	5 (33%)
Summer 1998/1999	10 (42%)	0 (0%)	10 (42%)	10 (42%)	4 (16%)	14 (58%)
Autumn 1999	8 (25%)	2 (6%)	10 (31%)	17 (53%)	5 (16%)	22 (69%)
Winter 1999	5 (23%)	4 (18%)	9 (41%)	9 (41%)	4 (18%)	13 (59%)
Spring 1999	1 (6%)	0 (0%)	1 (6%)	9 (56%)	6 (38%)	15 (94%)
Summer 1999/2000	3 (16%)	0 (0%)	3 (16%)	11 (58%)	5 (26%)	16 (84%)
Autumn 2000	6 (23%)	1 (4%)	7 (27%)	17 (65%)	2 (8%)	19 (73%)
Winter 2000	3 (17%)	0 (0%)	3 (17%)	13 (72%)	2 (11%)	15 (83%)
Spring 2000 ^a	6 (43%)	0 (0%)	6 (43%)	7 (50%)	1 (7%)	8 (57%)
Summer 2000/2001 ^b	12 (50%)	1 (4%)	13 (54%)	9 (38%)	2 (8%)	11 (46%)
Autumn 2001	6 (43%)	3 (21%)	9 (64%)	2 (14%)	3 (21%)	5 (36%)
Winter 2001 ^c	1 (6%)	2 (13%)	3 (19%)	8 (50%)	5 (31%)	13 (81%)

^{*} Winter 1998 was excluded from statistical analysis.

^a No trapping in October and November 2000, ^b no trapping in December 2000, ^c only trapping in June 2001.

Overall, higher proportions of seropositive females were observed in the peak breeding (spring) seasons of 1999 (31%), and 2000 (7%), but no females were seropositive in the spring of 1998 (Figure 6). Highest proportions of seropositive males tended to occur during the winter months, especially following the RHD outbreaks of 1999 and 2001 (Figure 6).

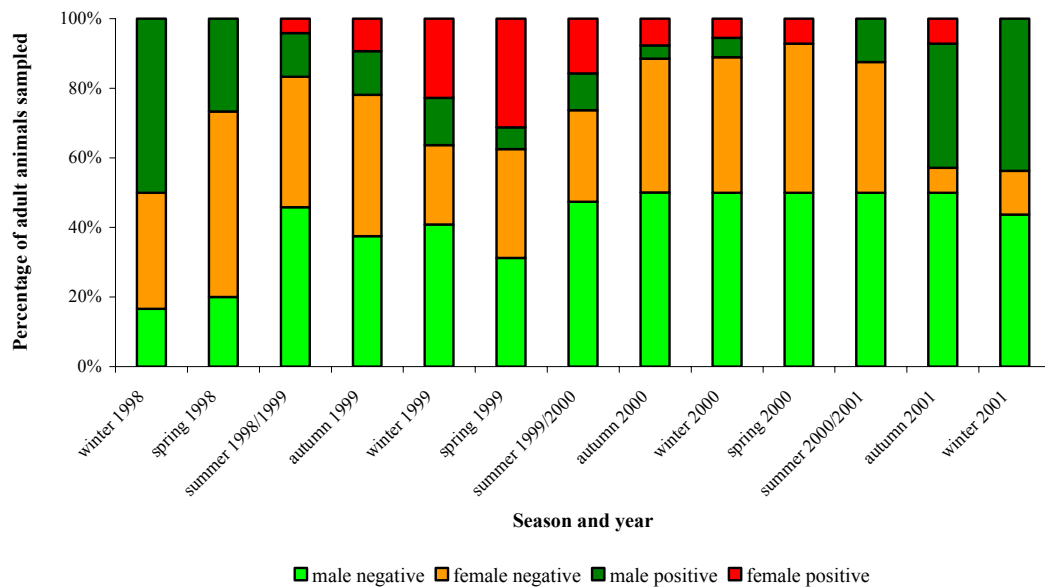


Figure 6: Serological status to RHDV of adult rabbits by season from winter 1998 to winter 2001 in Himatangi, New Zealand (no trapping in October to December 2000).

Based upon isotype ELISA results, the majority of seropositive rabbits on the site were animals that had survived outbreaks of disease (Figure 7). Evidence of very recent infection, based on high IgM titres, was observed in rabbits during the observed epidemics in March/April 1999 and February 2001. IgM was also detected in one rabbit in April 2000 when no confirmed RHDV mortalities were found. Antibody profiles consistent with seropositivity to an RHDV-like agent were found in one adult animal in December 1998 and two animals in April 1999. All three of these rabbits subsequently became RHDV seropositive based on the competition ELISA.

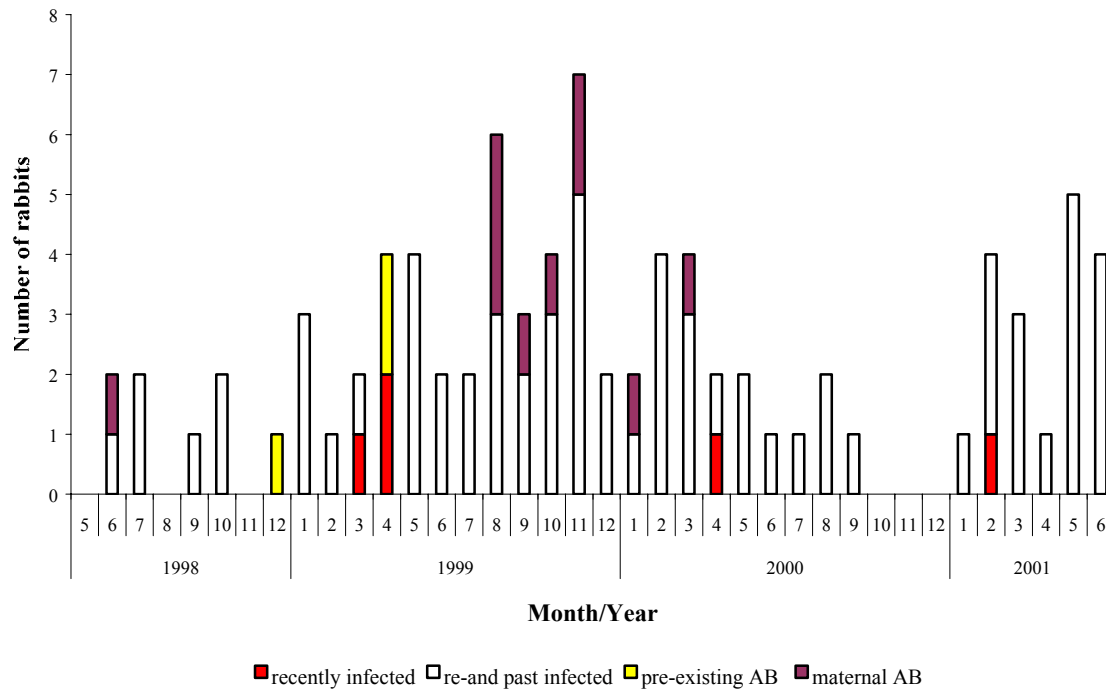


Figure 7: Number of seropositive rabbits categorised by immune status, based on isotype ELISA results in Himatangi, New Zealand between May 1998 and June 2001 (no sampling between October and December 2000, see Table 2 for definition of serological categories).

Serology of young (≤ 11 weeks) rabbits

During the entire study, 181 samples were obtained from rabbits less than 11 weeks of age. After exclusion of multiple samples from individuals within the same month, a total of 128 samples from 108 rabbits were included in the analysis. Of these rabbits, 12 animals (11%) were seropositive on the competition ELISA. In these rabbits, isotype ELISAs detected IgG, but not IgA or IgM, a profile consistent with the presence of maternally-derived antibody. The young rabbits with maternal antibody were temporally clustered, with 10 born during the breeding season following the 1999 outbreak (Figure 8).

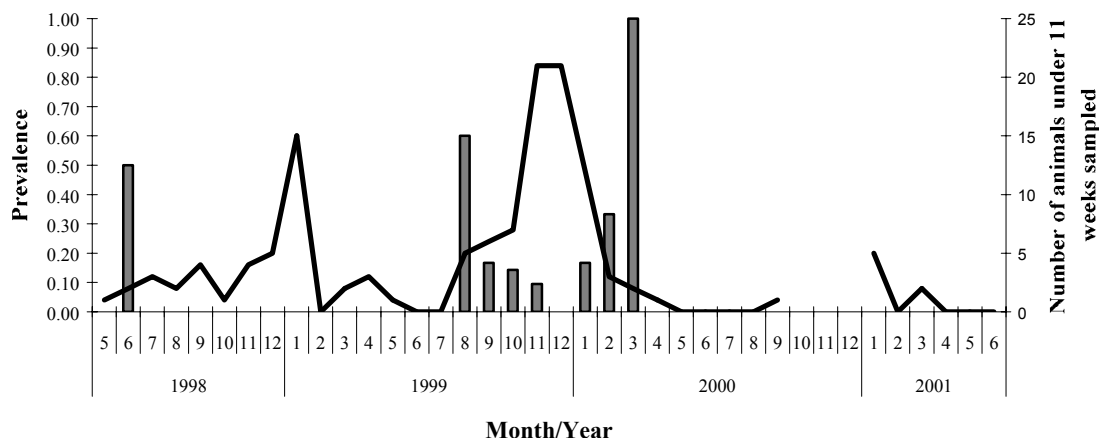


Figure 8: Number of immature rabbits (≤ 11 weeks) sampled monthly (line) and their temporal pattern of seroprevalence (bars) in Himatangi, New Zealand between May 1998 and June 2001.

Discussion

Rabbit population dynamics

Population size and migration

Owing to inherent difficulties in obtaining adequate numbers and representative samples, epidemiological studies in wildlife populations face problems of sampling bias beyond those of studies on domestic animal populations (Courchamp *et al.* 2000). With respect to determining the size of a study population, some authors have shown that estimation of rabbit population size is particularly problematic (Daly 1980; Smith *et al.* 1995). Traditional methods of capture-mark-recapture analysis (Begon 1979) for estimating population size in rabbits are inherently flawed because probability of capture is not homogeneous among adult rabbits but is influenced by factors such as age, sex, and time of the year (Daly 1980). Despite these limitations some researchers have employed capture-recapture methods (Dunnet 1957; Parer and Fullager 1986). We elected to combine intensive trapping with radio-tracking procedures in an effort to obtain more accurate estimates of population abundance and composition.

A large proportion (36.2%) of individuals were trapped for the first time as adults. These animals were either rabbits born at the study site that avoided trapping earlier in

life, or adult rabbits that immigrated from outside the study site. Most studies of rabbit populations have been conducted in enclosures which preclude the possibility of migration (Mykytowycz 1958; Myers 1958; Myers and Poole 1959; Gibb *et al.* 1978; Daly 1980; Webb 1993). The few studies conducted on open populations have provided minimal information about migration patterns (Dunsmore 1974; Gibb 1993; Gibb and Fitzgerald 1998). Daly (1980) considered adult rabbits to be sedentary and that the variation in the number of animals caught reflected variation in the trap response. He concluded that seasonal variation in trap response of adults could be due to mortality, trap saturation and sexual behavioural changes in the breeding season, but ignored the possibility of migration. Gibb (1993) reported the appearance of full-grown rabbits, and particularly males, of unknown provenance throughout the trapping period. Wood (1980) also reported this phenomenon, which he attributed wholly to rabbits born in the area avoiding capture as kittens, disregarding the possibility of immigration. Migratory behaviour in rabbits has obvious implications for transmission of infectious agents such as RHDV and also could influence temporal fluctuations in population immunity if migrants have different exposure histories to resident animals.

Consistent with previous studies, and owing to the intensity of trapping conducted, we consider that migration into the study site is a more probable explanation than avoidance of trapping for most of the rabbits first trapped as adults. This hypothesis is supported by the bimodal distribution of age at first trapping (Figure 1), the higher ratio of males among rabbits first trapped as adults, but not at less than 11 weeks, and the apparent difference in serological patterns of 'immigrant' compared with resident adults. All these observations point to the existence of two populations among animals at first trapping.

It is highly improbable that any rabbits trapped at less than 11 weeks of age would not have been born on the study site, and the distribution of age at first capture among these rabbits had a mode at 30 to 40 days, then declined noticeably. This distribution indicates that the majority of native-born rabbits would be trapped well before 11 weeks of age, and few rabbits would avoid capture beyond the minimum cut-off of 11 weeks used to categorise animals as immigrants. The predominance of males among rabbits first trapped as adults in this study and others (Dunsmore 1974) may be attributable to the larger home range of bucks (Henning *et al.* 2003b). This is consistent with the

findings of Myers and Poole (1961), who observed that movements of adult males were more numerous and widespread than those of females, although different mortality rates between sexes could also be involved. We interpret the collected information to reflect that immigration was not a trivial factor in the dynamics of this population and may have occurred to an extent that could contribute significantly to fluctuations in population immunity over time.

Temporal patterns in reproduction

Previous research in the North Island of New Zealand found breeding activity to be highest between June and November (Watson 1957). In the current study, pregnant rabbits were observed in all months of the year other than April, confirming that the rabbits bred for a large majority of the year. Gibb and Fitzgerald (1998), in a study of a sparse population on river flats, reported that breeding occurred throughout the year, except for autumn-winter, but that breeding could occur at all times if rabbits had access to improved pasture (as in the current study) (Gibb *et al.* 1969; 1985). The periods of maximum rabbit abundance (December to March) observed in this study are consistent with the findings of Gibb *et al.* (1978). Under the more extreme environmental conditions in the Central Otago region of New Zealand relative to the Manawatu, seasonality of breeding is more evident, and over 99% of pregnancies occurred within the period from September to February (Fraser 1988). Clearly, conditions of climate and feed availability will be major determinants of breeding activity and rabbit abundance from year to year.

A conspicuous feature of the data was the relatively high prevalence of pregnant females during several months in late 1999, and the consequent increase in numbers of young rabbits born on the site. However, this peak in pregnancies was not associated with any evident shift in the ratio of males to females. These events may best be explained by increased fertility in the breeding pairs. In females, marked seasonal variation (Watson 1957) and annual variation (McIlwaine 1962) in the number of ova ovulated have been described, and male fertility based on testicular position (abdominal or scrotal) fluctuates between consecutive months (Fraser 1988). Although climatic factors are considered to be important determinants of breeding activity and fertility

(Myers and Poole 1962), it is not possible to attribute the observed patterns to specific climatic events during this study (Gibb *et al.* 1978; Fraser 1988).

Mortalities

RHD outbreaks in 1999 and 2001 immediately preceded rapid and dramatic declines in rabbit abundance. A decline in rabbit abundance during mid-2000 was comparable in magnitude to the declines observed following RHD epidemics, albeit the reduction in population was more gradual. Mortalities at this time were attributed primarily to predation and there were no confirmed RHDV deaths (Henning *et al.* 2003b). The high risk of predation or scavenging of carcasses at this time may have restricted the availability of samples for PCR testing, and hence the ability to detect RHD mortalities. Hence, some contribution of RHD deaths at this time cannot be completely excluded. Reddiex *et al.* (2002) described an interaction between predation and RHD, where populations declined more rapidly after RHD outbreaks on sites where predators were present than on sites where predators had been removed.

Seroepidemiology

Test characteristics and cut-off selection

Reliable estimates of the sensitivity and specificity of serological tests for RHDV have proved difficult to obtain (McPhee *et al.* 2002), and different authors have selected different cut-off values for the Capucci competition ELISA employed in this study (Cooke *et al.* 2000; Zheng *et al.* 2002; Robinson *et al.* 2002a). All published authors (Capucci *et al.* 1997; Cooke *et al.* 2000; 2002; Zheng *et al.* 2002) agree that the titre of a sample is defined as the serum dilution at which the OD value of the negative control is reduced by 50%. MCPhee *et al.* (2002) estimated that 100% specificity was achieved with the competition ELISA at a cut-off of 50% in 1:10 serum dilution, but sensitivity was only 39%. The same authors observed that the performance characteristics might vary with host factors such as age, and between regions. Serological reactions at low titres are difficult to interpret and have been attributed to exposure to cross-reacting viruses, including an avirulent variant of RHDV (Capucci *et al.* 1997). Using the competition ELISA, Capucci *et al.* (1997) found low antibody titres in a commercial rabbitry which he attributed to cross-reaction following infection with a non-pathogenic

RHDV-like rabbit calicivirus (RCV). Experimental infections with RCV resulted in antibody titres in the competition ELISA that were 10-20 fold lower than those of RHDV-convalescent rabbits (Capucci *et al.* 1996). Low titres of antibody in the competition ELISA in samples from wild rabbits collected before the introduction of RHDV in New Zealand (O'Keefe *et al.* 1999) and Australia (Cooke *et al.* 2000; Robinson *et al.* 2002a), in association with high IgG titres (Cooke *et al.* 2000), were also interpreted as evidence for infection with a non-pathogenic calicivirus. These titres appear to have some protective effect against RHDV and are clustered in wetter regions of Australia, providing a potential explanation for the lower impact of RHDV in these areas (Cooke *et al.* 2002).

Although O'Keefe *et al.* (1999) detected antibodies against RHDV in New Zealand rabbits before the virus was introduced, based on the high mortality rates reported after the release of the virus it appears that these antibodies did not protect against RHD epidemics. The origin of these reactions is not known. Because false positive results have been documented in wild rabbits (Cooke *et al.* 2002; Robinson *et al.* 2002a), in line with Parkes *et al.* (2002) we adopted a conservative approach, and only titres greater or equal to a 1:40 dilution in the competition ELISA were deemed positive for RHDV. With respect to the possibility of an RCV-like virus occurring at the site, we used the criteria of Cooke *et al.* (2002); if the serum profile of the competition ELISA had a titre of $\leq 1:10$, there was no IgA and IgM, and the IgG titre was high ($\geq 1:640$), then this was considered indicative of an RCV-like infection.

Approaches to interpretation of the isotype ELISAs have also varied. Cooke *et al.* (2000; 2002) referred to OD values to the positive and negative control reference samples rather than the OD value of the serum control (serum sample at 1:40 without adding RHDV antigen to the well) used in this study and previously by Zheng *et al.* (2002). We assigned rabbits to serological categories based on the estimated titres, which retains more information than dichotomous (positive or negative) results (B. Cooke pers. comm.).

High titres (1:640) of IgM occur within 5 days after inoculation of rabbits with RHDV (Henning, unpublished data), peak within 2 weeks and decline quickly (Cooke *et al.* 2000). IgA titres decline more slowly than IgM, and IgG titres can persist for

several months (Cooke *et al.* 2000). Therefore, the ability to identify recent, past and re-infection events based on loss of IgM and IgA depends upon frequent capture and sampling. If trapping is infrequent it is difficult to discriminate between past infections and re-infection. For this reason, the suggested categories of past infection and re-infection, based on Cooke *et al.* (2000) and shown in Table 2 were combined to form a single category (Figure 7).

Evidence of an RCV-like virus

Throughout our study, only three rabbits sampled in late 1998 and early 1999 had serological profiles consistent with an RCV-like infection. However, not all samples that were seronegative in the competition ELISA were tested using the isotype ELISAs. These three rabbits were all born in mid-1998, and showed serological profiles indicating a protective immune response that was similar to profiles of rabbits infected experimentally with RCV (Capucci *et al.* 1996). All three had IgG titres of 1:640 prior to seroconverting in the competition ELISA. Rabbits with high titres of cross-reactive antibodies have a higher probability of surviving RHD outbreaks (Nagesha *et al.* 2000). Although we consider the influence of an RCV-like infection on RHD epidemiology in our study was probably negligible, the origin and importance of serological reactions to RCV-like viruses in New Zealand warrants further investigation.

Maternally derived antibodies

Resistance to RHDV infection in young rabbits is attributable to either innate resistance or maternally derived antibodies. Robinson *et al.* (2002b) modelled survival of kittens born to seronegative does, and predicted 90% survival for rabbits exposed to RHDV at 4 weeks of age compared with 10% survival for rabbits exposed at about 9 weeks of age. Cooke *et al.* (2000) claimed that maternal antibodies should be exclusively IgG (i.e. no IgA and IgM), and only persist in rabbits up to 11 weeks of age (mean 8 weeks). At the cut-off value at the 1:40 dilution that we adopted, the prevalence of maternal immunity in our study was very low (11%) among rabbits sampled at less than 11 weeks of age and assumed born on the study site. Thus, most young rabbits were probably susceptible to new RHD epidemics throughout most of the study period. Most rabbits with maternal antibodies were observed in late 1999, corresponding with

the peak of offspring born after the 1999 epidemic. The 2000/2001 breeding season did not produce any kittens with maternal immunity, probably as no RHD epidemic occurred in 2000. Had animals in this age range, beyond the age of innate resistance to infection and without maternally derived antibody been exposed to RHDV, then it should have resulted in disease (Smid *et al.* 1991; Morisse *et al.* 1991).

Parkes *et al.* (2002) speculated that offspring of does surviving RHD may become infected and immune without clinical disease provided that ‘the right dose at the right age’ was transmitted from the does to their offspring. Our longitudinal data revealed that high antibody titres (and possibly protection) following infection in this age group of rabbits may be transient. However, low maternal antibodies in kittens may be undetectable in the competition ELISA, but still protective (Robinson *et al.* 2002b). In an experimental study, Parkes *et al.* (2002) showed that kittens born to seropositive does, but seronegative at the time of first challenge, survived oral inoculation at the age of 9 weeks, but mostly died at 17 weeks after another challenge. Robinson *et al.* (2002b) also implied that viral replication may be suppressed if rabbits are exposed when the maternal antibody titre is higher than 1:40, and such animals may become susceptible to later challenge after antibody loss. It has been also suggested that seroconversion and persistent protection is induced if maternal antibody titres are lower than 1:40 at the time of challenge. The absence of detectable maternal antibody in young rabbits at most times, other than in the immediate wake of epidemics of RHDV induced mortality, is likely to be one factor contributing to the apparent cyclical patterns of mortality, that we observed.

Seroconversion in adult rabbits surviving epidemics

Seroprevalence in adult animals following outbreaks was between 40 and 50%, which is comparable to observations by Parkes *et al.* (2002) in Otago (30-45%). The overall proportion of adult seropositive rabbits over the study period was 20% (77 seropositive adult animals of 385 monthly samples), which was again similar to New Zealand observations in cross-sectional studies in Central Otago (Earnscliffe: 28%) and the Mackenzie Basin (19%), but lower than a prevalence of 55% in North Canterbury (Parkes *et al.* 2002). Direct comparisons to Australian sites are difficult to

conduct, as most researchers use the 1:10 dilution of sera, to consider samples to be positive (Mutze *et al.* 2002).

High post-epidemic seroprevalence was observed for seven months in 1999 and preceded the peak in maternal antibody prevalence described in young rabbits. Despite the fact that most adult animals were seronegative in autumn 2000, and the presence of one rabbit at that time with IgM (indicating recent infection), no mortalities were attributable to RHDV nor was there evidence of significant seroconversion. Better understanding of the relationship between ELISA titres and protective immunity would aid explanation of the prolonged inter-epidemic intervals and apparent cyclicity of the disease.

Declining titres were observed in only 17% of adults and loss of detectable antibody in seropositive adult animals appears to be a relatively minor factor influencing shifts in population immunity. The decrease in seroprevalence in 2000 was mainly attributable to i) a steady influx of seronegative adult animals, which may not have had contact with RHDV and ii) the loss of maternal antibody in young adults. The influx of seronegative animals was greater in the 1999 and 2001 breeding seasons preceding the RHD outbreaks than in 2000. This supports the hypothesis that fluctuation in population immunity is a key determinant of the temporal occurrence of RHDV outbreaks in a sparse population, and that both immigration and population turnover are key factors.

Overall, we conclude that RHDV persisted in this population in the absence of deliberate re-release of the agent. Outbreaks were intermittent and associated with sharp declines in rabbit abundance. However, comparable declines in the population may occur due to competing risks of mortality, particularly predation, at different times. Similar observations were made in France (Marchandeau *et al.* 2000), where following initial outbreaks of RHD in 1995 and 1996, seroconversion in the absence of mortalities occurred in subsequent years, suggesting the establishment of effective population immunity in the face of persistent challenge. Marchandeau *et al.* (2000) further hypothesised that predation (by red foxes) may have constrained population recovery following outbreaks as rabbit numbers remained low for a period of three years.

The probability of propagated epidemics of RHD mortality will be determined by fluctuations in population dynamics and the proportion of susceptible individuals, as well as factors influencing the probability of effective exposure and transmission. Contributing factors may include climate, which influences the abundance of flying vectors (Henning *et al.* 2003c), and breeding patterns (Saunders *et al.* 1999; Mutze *et al.* 2002). RHDV is generally less effective in high-rainfall, cooler, wetter areas in Australia (Saunders *et al.* 1999; Henzell *et al.* 2002). Based on comparison of our data collected in the Manawatu with observations in the drier region of Otago (Parkes *et al.* 2002) this may be also true in New Zealand. This could be attributable to cross protection from related viruses (Cooke *et al.* 2002) or other factors such as viral persistence in the population. The persistence of virus in the environment appears to be limited to a few months (Henning *et al.* 2003d), but the reservoirs of viral persistence in the inter-epidemic periods are not well defined and the potential role of carrier rabbits needs further investigation (Shien *et al.* 2000; Zheng *et al.* 2002).

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Typical position of a rabbit succumbing to rabbit haemorrhagic disease



Rabbit carcass found after cat predation



Ulex europaeus



Cytisus scoparius



Cortaderia sp.

Chapter 3

Spatial and temporal dynamics of mortality and home range use in wild rabbits (*Oryctolagus cuniculus*) with an emphasis on rabbit haemorrhagic disease (RHD)*

*Submitted by J. Henning, D. U. Pfeiffer, P. R. Davies and M. A. Stevenson to *Wildlife Research*

Abstract

A sparse rabbit population in New Zealand was intensively monitored over three years to assess the temporal and spatial dynamics of rabbit mortality. A total of 107 deaths were recorded, of which 93 could be classified by cause. The predominant cause of mortality was predation (49% of deaths), followed by rabbit haemorrhagic disease (20%). Deaths due to rabbit haemorrhagic disease (RHD) were clustered in time (within three weeks) and in space, predation occurred most actively from late autumn to spring while other causes of death did not show pronounced seasonal peaks. No differences in cause-specific death risk were observed between sexes. Deaths due to predation were most common for younger animals, while RHD mortality occurred mainly in older rabbits. Kernel density estimators and minimum convex polygons were used to calculate core and home ranges of rabbits, and estimated least squares geometrical means of core range and home range were adjusted for sex and season. Core ranges were larger for males than for females in winter and the home ranges of male rabbits were significantly larger in the winter and spring season compared to female home ranges. Rabbits with RHD died closer to their home range centroid than animals dying from other causes. We conclude that rabbit control by spread of *Rabbit haemorrhagic disease virus* (RHDV) contaminated baits might be more successful when distributed close to burrow entrances or to surface cover used by rabbits as denning sites.

Introduction

Studies of mortality in animal populations usually define a population of individuals-at-risk, and determine the reasons and timing of death events occurring during a defined follow-up period. Such studies define both reasons for mortality and periods of time when risk of death is greatest (McLennan *et al.* 1996; McLellan *et al.* 1999; Robertson and Colbourne 2001; Taylor *et al.* 2002; Brown and Sleeman 2002). Whereas in most animal populations the goal of this type of research is to identify risk factors for mortality and to decrease losses, for pest populations the goal is the opposite: to identify risk factors for death that can enable control activities to be optimised, thereby minimising the destructive capacity of these pest populations at an acceptable cost.

The use of radio telemetry has provided a major advance in the study of mortality in wild rabbit (*Oryctolagus cuniculus*) populations, allowing carcasses to be retrieved for a more precise diagnosis of death (Richardson and Wood 1982; Robson 1993; Moriarty *et al.* 2000; Reddiex *et al.* 2002). Integrated studies of cause-specific mortality and area use by rabbits are non-existent in the literature. However, this approach has obvious applications with regards to infectious diseases such as rabbit haemorrhagic disease (RHD), since home range usage is likely to influence spatial patterns of RHD outbreaks.

This is the second paper in a series examining the epidemiology of RHD in an open population of wild rabbits. Here we used radio-tracking techniques to monitor a population of rabbits over a 38-month period to firstly describe cause-specific mortality risks, and secondly describe the spatial and temporal patterns of mortality.

Materials and Methods

Study area

The study area was located near Himatangi (longitude: 175.300, latitude: -40.425), 30 kilometres west of Palmerston North in the lower North Island of New Zealand. The study area was comprised of 30 hectares of dense vegetation and pasture farmland, as described previously (Henning *et al.* 2003a). The period of study was from 1 May 1998 to 30 June 2001. Throughout this period the study area was visited three times per week during day-time and at each visit, trapped rabbits were sampled and the site traversed for 2 to 12 hours using a radio-tracking device to identify individual rabbit locations.

Radio-tracking of rabbit locations

A series of 40 marker points was established throughout the study area, consisting of either 3.5 metre wooden pillars ($n = 12$) or prominent features of the landscape (trees, tree stumps and fence corners, $n = 28$). At the start of the study the exact location of each marker location was determined using a Global Positioning System (GPS) device (Trimble Navigation Ltd., Sunnyvale, CA, USA). Cage traps, incorporated in a fence line between known densely vegetated denning areas and pastoral feeding areas (Henning *et al.* 2003a), were used to trap rabbits as they joined the resident population (immigrants) and to monitor those animals already present in the study area. At each

visit to the study site trapped animals were anaesthetised, sexed and weighed and a blood sample was collected for testing for antibodies to RHDV (Henning *et al.* 2003a). Animals trapped for the first time were ear tagged and fitted with a radio collar using a plastic cable tie collar to attach the radio to the animal's neck. Two types of radio collars were used: Titley collars (Titley Collars, Waipara, New Zealand) and Sirtrack collars (Sirtrack Limited, Havelock North, New Zealand). Both types of collars incorporated a motion link device, which generated a higher pulse rate after a set period of inactivity.

Animals were tracked using a portable radio telemetry receiver (Model CE 12, Custom Electronics of Urbana Inc., Urbana, Illinois, USA) and a three-element yagi antenna. Radio-tracking was conducted to localise an animal within one to two metres, and if possible to sight it. For animals identified at each tracking session the distance (in metres) to the nearest marker point was estimated visually and the direction to the marker point estimated using a Recta DP 6 Swiss army compass. Animals were located a minimum of once a week and typically several times a week. Time of the day was classified as day or dusk depending on the light conditions (Gibb 1993), and the vegetation characteristics of each location were recorded.

Rabbits that had died since the previous visit were located (on the basis of a characteristic inactivity tracking signal) and retrieved for post mortem examination. Reasons for death were based on post mortem examination and histology of organ samples. *Rabbit haemorrhagic disease virus* (RHDV) was detected using reverse transcriptase polymerase chain reaction (RT-PCR) of liver tissues (Nowotny *et al.* 1997). Criteria for assigning cause of death are shown in Table 5. At the end of the study, all members of the resident population were trapped and euthanised. Liver samples were retrieved from this group and submitted for RHDV PCR.

Table 5: Criteria used to assign causes of rabbit death

Reason	Criteria
Rabbit haemorrhagic disease	Characteristic gross pathological signs, confirmed by histology of tissue samples and positive RHDV PCR in liver tissue.
Predation	Typical predation patterns recorded, as described below. Predation by certain species usually confirmed by trapping the same mammalian predator species within 2-3 days of each rabbit death (Henning <i>et al.</i> 2003b). No RHDV detected by PCR of liver tissue. Criteria for determining predator species as follows: Cat: body opened and one or more long bones broken; internal organs often removed (frequently only stomach remained); muscle flesh eaten; head removed or ears eaten; often only remains of skin, which is regularly ‘pulled over the legs’; bite marks typically 3–4 cm apart. Stoat or ferret: large long bones usually not broken; internal organs completely or partly eaten; neck broken between cervical vertebrae III and VI; muscle flesh never completely eaten; bite marks less than 2 cm apart. Hawk: carcass surrounded by fur; internal organs eaten or spread in a circle 20-50 cm in diameter; eyeballs usually removed.
Other natural causes	Death without signs of predation or accident. PCR on liver tissue was negative for RHDV.
Accidents	Death occurring as a result of conduct of the study.

Statistical analyses

Cause-specific mortality

The Chi-squared test was used to test for differences in the gender composition of each cause of death.

Temporal patterns of mortality

Cause-specific rates of death were described in terms of incidence density (Thrusfield 1997). Here, the numerator was the number of animals succumbing to a given cause of death (RHD, predation, and other natural causes). Estimates of rabbit abundance were used to determine the denominator, the total number of rabbit days at risk (Henning *et al.* 2003a). Incidence density was described as the number of deaths per 1,000 rabbit-days at risk.

On the basis of weight changes (recorded at each trapping event), a Gompertz growth model was derived and individual animal birth dates were estimated (Henning unpublished data; Cowan 1983; Webb 1993). Age at death was described using a

survival analysis approach where the outcome of interest was age (in days) at death for each of the three assigned natural death reasons. Animals still alive at the end of the study period or which lost their radio collar were treated as censored observations. A competing risks approach was used to characterise cause-specific mortality patterns as a function of rabbit age (Marubini and Valsecchi 1996). Under the assumption that there was independence among the different reasons of death, a log rank test was used to test for differences in ages at death for each of the three natural death reasons.

Core and home range estimation

The Geographic Information System (GIS) software ArcView for Windows Version 3.1 (Environmental Systems Research Institute, Inc. Redlands, CA, USA) was used to plot trap and animal sighting locations. Marker locations were plotted in the GIS and the recorded distance and direction of each rabbit sighting from individual reference markers were used to estimate the precise location of each sighting event. Animal sighting locations were used to describe core ranges and home ranges. Core ranges, representing the main denning areas used during the day, were estimated based on day-time radio-tracking locations. Home ranges, comprised of the core range plus an additional area representing the area of grazing and social interaction were estimated from data obtained from trapping locations in addition to all radio-tracking events (including those obtained at dusk).

Core and home ranges were estimated using two methods: i) minimum convex polygon (MCP) techniques (Mohr 1947), and ii) kernel density estimation techniques (Worton 1989). The MCP method combines all peripheral points with every internal angle not exceeding 180 degrees. Kernel density methods use accumulated point locations to determine the area of highest individual rabbit sighting density. Fixed smoothing parameters were used for the kernel density estimation method, selected by least squares cross-validation. Core and home ranges were described in terms of contour maps with contour lines identifying the probability of finding an animal at a given location. Probabilities of animal locations (50% and 95% isopleths) were used to divide the area into use intensity, with the 95% isopleth representing the area within which an animal could be located on 95% of occasions (Ford and Krumme 1979).

Analyses were conducted to determine the minimum number of radio-tracking events necessary to estimate core and home range sizes. This was determined graphically by plotting estimated home range size as a function of the number of radio-tracked events (Harris *et al.* 1990). The number of tracking fixes required to describe core range was that at which the core range size reached an asymptote. The cumulative percentage of core range size did not reach complete stabilisation in the asymptote, but increases in range sizes were minimal after 7 radio-fixes. As ranges were anticipated to vary between seasons, only animals with more than 7 radio-fixes per season were used for the analyses presented.

A general mixed marginal model (PROC MIXED macro in SAS, The SAS System for Windows, Release 8.2, SAS Institute Inc., Cary, NC, USA) was used to determine the least squares geometric mean estimate of core range and home range area (termed the geometric least squares mean in the discussion that follows). This measure uses an exchangeable correlation structure and provides an estimate of core range and home range area adjusted for the influence of sex, season, and repeated observations made on the same animal over time (Diggle *et al.* 2002).

A generalised linear model approach was used to compare the natural logarithm of distances between death location and home range centroid for each of the three death categories. Multiple comparisons were conducted using Fisher's least significant difference test (LSD).

Spatio-temporal clustering of RHD deaths

The presence of spatio-temporal clustering of RHD deaths during the study period was assessed using the space-time scan statistic (Kulldorff and Nagarwalla 1995). Adopting a case-control approach, RHD deaths were assigned as cases, and deaths for all other reasons were assigned as controls. The time and place of RHD deaths was assumed to follow a Bernoulli distribution and the null hypothesis was that the risk of a rabbit death being due to RHD was the same throughout the study area. The space-time scan statistic imposes a 'cylinder' centred on each of the identified death locations. For each death location, the radius of the window is set to vary continuously in size from zero to an upper limit of not greater than 50% of the total area under investigation. The

height of the cylinder is set to vary according to time. A large number of distinct cylinders are created, each with a different set of rabbit death events within it, and each cylinder is a possible candidate for delineating a spatio-temporal cluster of RHD deaths. The method tests the null hypothesis that there is the same risk of RHD deaths occurring within the current cylinder when compared with those deaths outside of the cylinder (Kulldorff 1997).

Results

Cause-specific mortality

Throughout the 38-month study period, 107 rabbit deaths were recorded. A total of 86 carcasses were found on the basis of a radio-signal frequency indicative of mortality; seven rabbits were found dead when trapped. At the end of the study, radio signals were used to locate and humanely euthanase 14 radio-collared rabbits. Liver samples were retrieved from this group and all were negative for RHDV PCR. A summary of data on body condition at death is shown in Table 6.

Table 6: Classification of body condition at death for 107 rabbit deaths examined in a 38 month observational study of rabbit mortality in a 30 ha rural area in Himatangi, New Zealand

Carcass condition	Number (%)
Carcass in good condition. Body cavity intact and all internal organs present.	48 (45%)
Carcass partially eaten. Abdomen or chest cavity opened and internal organs removed.	17 (16%)
Carcass mostly eaten. Internal organs removed from the abdomen or chest cavity.	36 (33%)
Skin, bones and only small amounts of internal organs remaining.	
Carcass showing no signs of being scavenged although autolysed.	3 (3%)
Carcass showing no signs of being scavenged. Body cavity intact, although dehydrated.	3 (3%)

Counts of rabbit deaths and descriptive statistics of age at death, stratified by cause of death are shown in Table 7.

Table 7: Summary of mortality causes and descriptive statistics of estimated age at the time of death for 93 rabbit carcasses

Cause of death	Number (%)	Estimated age at death (days)		
		Mean (SD)	Median	Q1, Q3
Rabbit haemorrhagic disease:	18 (20%)	280 (211)	202	152, 358
Predation:				
Cat	37 (40%)			
Stoat or ferret	5 (5%)			
Hawk	2 (2%)			
Total deaths from predation	44 (47%)	252 (178)	207	127, 335
Other natural causes:				
Pneumonia	5 (5%)			
Kidney failure	2 (2%)			
Intestinal bleeding	1 (1%)			
Coccidiosis	1 (1%)			
Undiagnosed	9 (10%)			
Total deaths from other natural causes	18 (19%)	327 (300)	282	71, 410
Accidental deaths:				
Anaesthesia ^a	3 (3%)			
Trapping ^b	5 (6%)			
Radio collar ^c	2 (2%)			
Other interventions	3 (3%)			
Total accidental deaths	13 (14%)	245 (262)	214	70, 245

^a Deaths that had occurred under anaesthesia, representing 0.35% of all anaesthetic procedures conducted.

^b Deaths associated with trapping, representing 0.5% of all trapping events.

^c Deaths associated with application of radio collars.

A total of 34 females and 46 males died from RHD, predation or other natural causes of death. The proportion of deaths attributed to RHD did not differ between sexes (females: 7 of 34; males: 11 of 46; $\chi^2 = 0.0003$; $df = 1$; $P = 0.99$). Similarly, there were no differences between sexes for deaths attributed to predation (females: 18 of 34; males: 26 of 46; $\chi^2 = 0.0003$; $df = 1$; $P = 0.99$) or to other natural causes (females: 9 of 34; males: 9 of 46; $\chi^2 = 0.1$; $df = 1$; $P = 0.75$).

Temporal patterns of mortality

Mortality as a function of calendar time

Line plots showing the incidence density of death as a function of calendar time, stratified by each of the natural causes of death (RHD, predation and other natural causes) are shown in Figure 9.

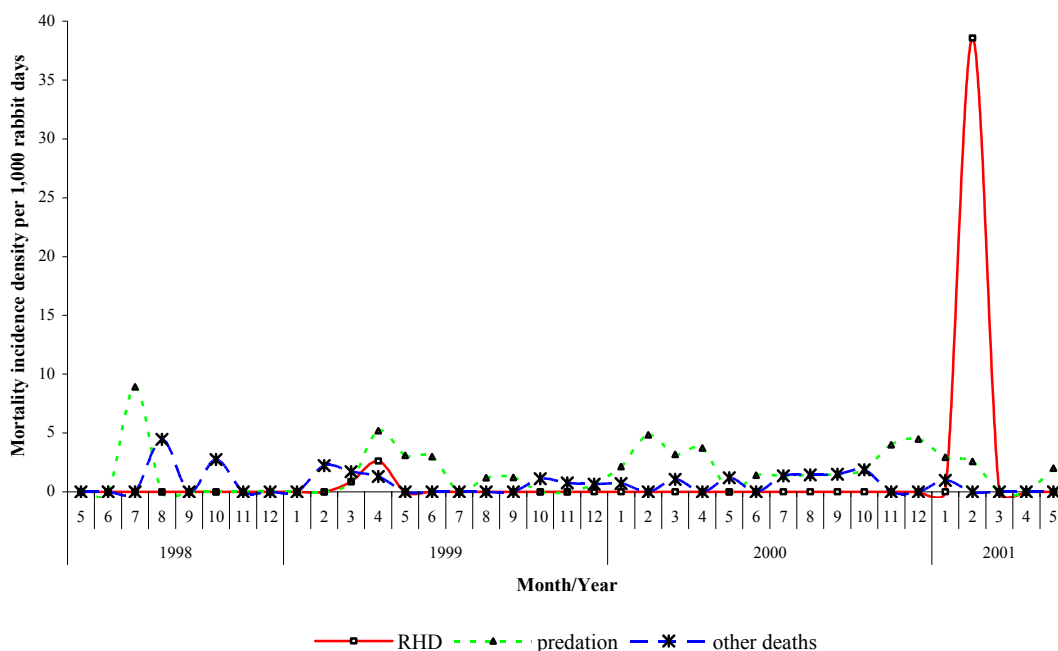


Figure 9: Cause-specific incidence density of mortalities (expressed as deaths per 1,000 rabbit-days at risk) as a function of calendar time for the three major natural mortality causes recorded in this study: RHD, predation and other natural causes.

The first RHD outbreak on the study site, confirmed by detection of RHD in the liver tissue from a carcass retrieved in March 1998, occurred before the official start of the study. The first mortality peak of the study was a high predation peak observed in July 1998, followed by a peak in deaths from other natural causes in August and October 1998. In 1999 there was a relatively small increase in the incidence density of RHD during March and April. In 2000, no carcasses were found to be positive for RHDV and predation was the major reason for death during the late summer and autumn months (February through to April 2000). Predation declined over the winter months, and then increased again in the spring and early summer (September through to December 2000). In general, the incidence density of deaths due to predation peaked over the summer and

autumn period. In February 2001, a second RHD outbreak occurred, resulting in the highest mortality rate observed over the entire study period. RHD deaths, when they occurred, were temporally clustered within time frames of three to four weeks (Figure 10).

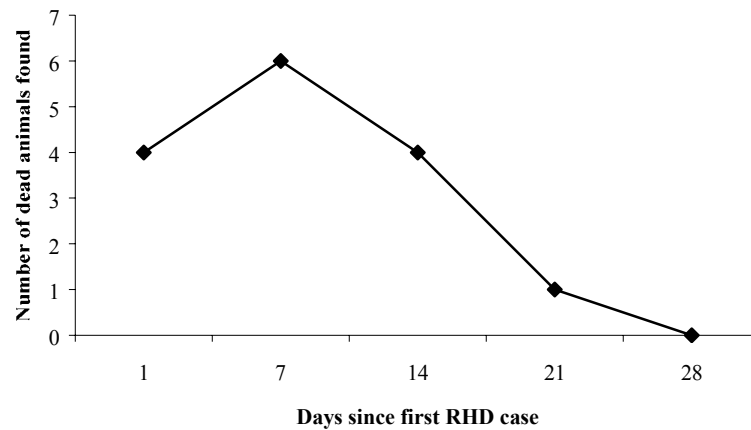


Figure 10: Number of animals dying from RHD in seven day intervals during the 2001 RHD epidemic.

Mortality as a function of animal age

Kaplan-Meier survival curves showing the cumulative proportion of rabbits that remained alive, as a function of age and stratified by the three natural death reasons are shown in Figure 11. Predation resulted in a high rate of mortality among rabbits of young age and accounted for the greatest number of deaths. Differences in the age of death were significant among death categories (log rank test statistic = 16.9; $df = 2$; $P < 0.0001$).

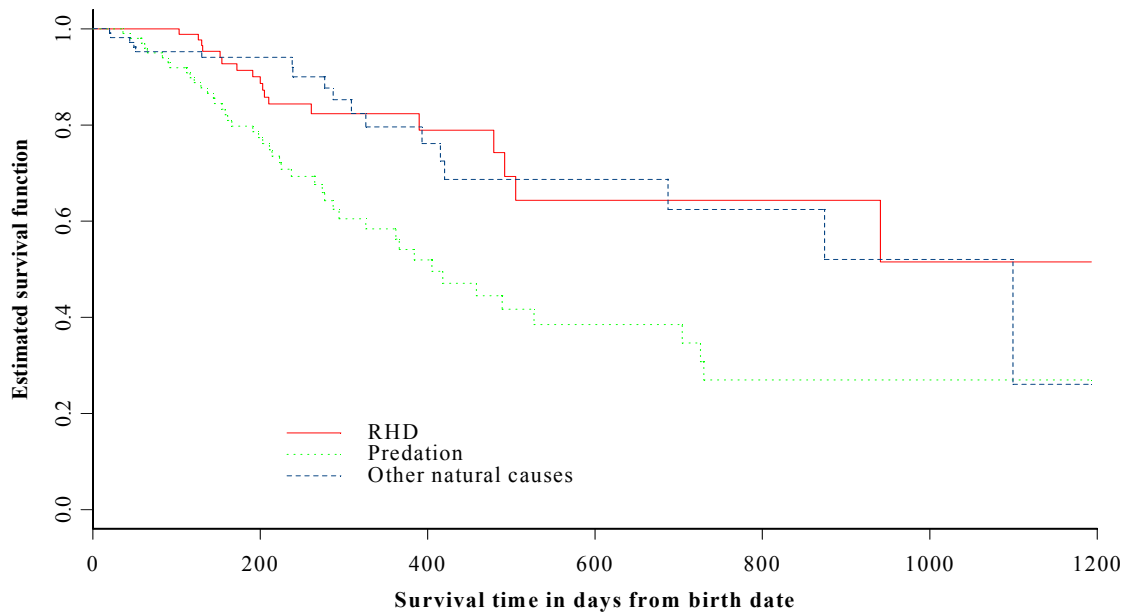


Figure 11: Kaplan-Meier survival curves showing the estimated survival as a function of age stratified by the three natural causes of death (RHD, predation, and other natural causes).

Core and home ranges

A total of 1,021 radio-tracking records were obtained between 15 August 1998 and 27 June 2001. Of this total, 945 records were used in the analyses presented. The remaining records were excluded on account of data entry errors, recording errors or insufficient information about individual animals tracked. Of the records used for analysis, 825 were obtained during daylight hours and 120 were obtained at dusk.

A total of 105 animals (67 males and 38 females) were radio-tracked throughout the study period and the median number of tracking events per animal was 4 (95% CI 1-36). Individual animals were radio-tracked for a maximum of 942 days in core areas (median 207 days, 95% CI 12-788 days) and the median interval between radio-tracking events for calculation of core areas was 7 days (95% CI 1-34 days).

For home range estimations, rabbits were monitored for a maximum of 955 days (95% CI 0–724 days) and the median interval between consecutive home range localisations of the same animal was 7 days (95% CI 0–34 days).

Box-and-whisker plots showing the distribution of observed home range areas (based on the 95% isopleth of the kernel density estimation), stratified by sex and season are shown in Figure 12.

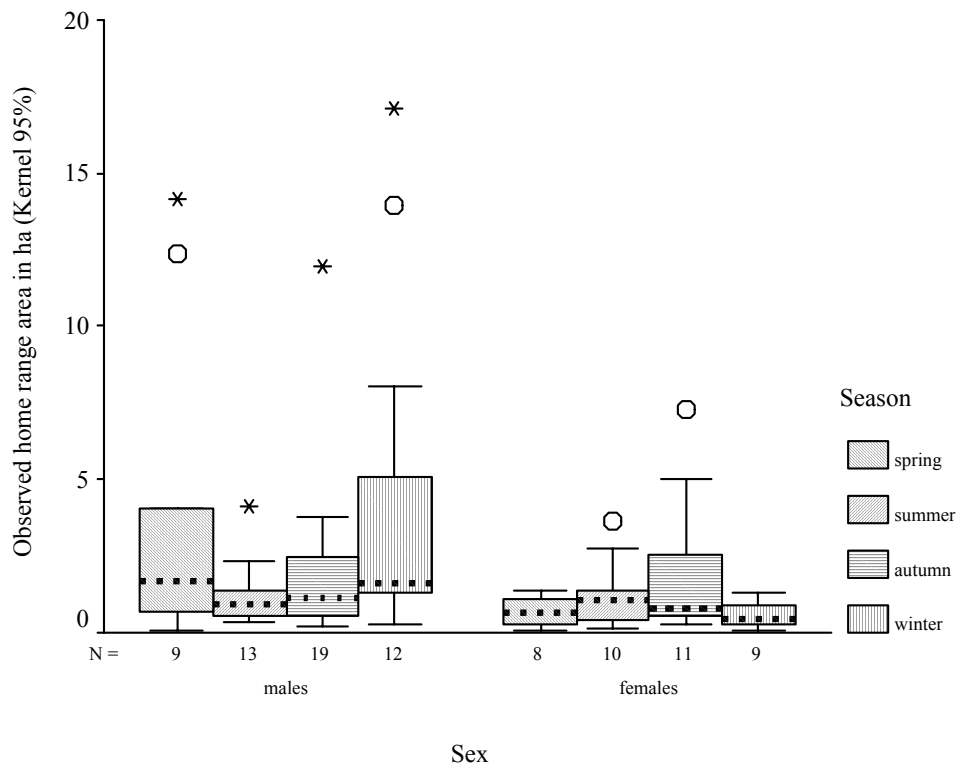


Figure 12: Box-and-whisker plots of the distributions of observed home range size (ha) based on 95% kernel density estimation. Home range areas were estimated for rabbits with greater than 7 tracking events per season. Outlier (°) and extreme values (*) are displayed. The dotted bar indicates the median.

Observed home range areas were highly skewed, and for this reason the geometric least squares means have been used to describe home range and core range areas.

Core range size

Geometric least squares mean core range estimates are shown in Table 8. Core range areas were larger for males than females, but the difference between sexes was not significant for any of the three calculation methods used when analysed independently from season (results not shown). Males had the smallest core range area during the

autumn and females had the smallest core range area during winter. Winter core ranges for males were significantly larger than those for females for all three methods of calculation (Table 8).

Table 8: Geometric least squares mean (Geo-LSM) of estimated core range area (ha) stratified by sex and season for rabbits radio-tracked during daylight hours between autumn 1999 and winter 2001. Core ranges were estimated using kernel density and mean convex polygon (MCP) methods

Sex	Season	n	Geo-LSM (95% CI)		
			kernel 50%	kernel 95%	MCP
Male	Spring	6	0.23 (0.08 - 0.73)	1.20 (0.36 - 4.01)	0.38 (0.11 - 1.34)
	Summer	3	0.17 (0.04 - 0.78)	0.83 (0.16 - 4.33)	0.29 (0.05 - 1.63)
	Autumn	5	0.11 (0.03 - 0.41)	0.78 (0.20 - 3.00)	0.23 (0.06 - 0.94)
	Winter	10	0.30 (0.12 - 0.77) ^a	1.65 (0.62 - 4.39) ^b	0.52 (0.19 - 1.41) ^c
Female	Spring	4	0.07 (0.02 - 0.28)	0.41 (0.09 - 1.77)	0.17 (0.04 - 0.77)
	Autumn	8	0.18 (0.04 - 0.87)	1.26 (0.23 - 6.84)	0.36 (0.06 - 2.10)
	Winter	9	0.05 (0.02 - 0.16) ^a	0.31 (0.10 - 0.93) ^b	0.12 (0.04 - 0.36) ^c

CI: confidence interval.

^a Test of fixed-effects least squares mean significantly different (t statistic = 2.62, df = 11, $P = 0.024$).

^b Test of fixed-effects least squares mean significantly different (t statistic = 2.51, df = 11, $P = 0.029$).

^c Test of fixed-effects least squares mean significantly different (t statistic = 2.20, df = 11, $P = 0.050$).

Home range size

Geometric least squares mean home range estimates are shown in Table 9. Home range areas were larger for males than females, with the difference between sexes being significant ($P < 0.05$) for both kernel 50% and kernel 95% estimation methods (results not shown). Males had the smallest core range area during the summer and females had the smallest core range area during winter and spring. Males had significantly larger ($P < 0.05$) home range areas than females in winter and spring for kernel 50% and only in winter for kernel 95%, but not for MCP (Table 9).

Table 9: Geometric least squares mean (Geo-LSM) of estimated home range area (ha) stratified by sex and season for rabbits radio-tracked during daylight hours between autumn 1999 and winter 2001. Home ranges have been estimated using kernel density and mean convex polygon (MCP) methods

Sex	Season	<i>n</i>	Geo-LSM (95% CI)		
			kernel 50%	kernel 95%	MCP
Male	Spring	9	0.30 (0.13 - 0.69) ^a	1.37 (0.61 - 3.07)	0.43 (0.17 - 1.14)
	Summer	13	0.16 (0.08 - 0.31)	1.05 (0.53 - 2.07)	0.39 (0.17 - 0.88)
	Autumn	19	0.26 (0.14 - 0.46)	1.58 (0.90 - 2.78)	0.61 (0.31 - 1.19)
	Winter	12	0.35 (0.17 - 0.72) ^b	2.15 (1.06 - 4.34) ^c	0.55 (0.24 - 1.27)
Female	Spring	8	0.08 (0.03 - 0.18) ^a	0.47 (0.20 - 1.11)	0.19 (0.07 - 0.52)
	Summer	10	0.13 (0.06 - 0.29)	0.91 (0.41 - 1.99)	0.24 (0.10 - 0.62)
	Autumn	11	0.20 (0.09 - 0.43)	1.09 (0.51 - 2.30)	0.48 (0.20 - 1.16)
	Winter	9	0.06 (0.03 - 0.15) ^b	0.42 (0.19 - 0.95) ^c	0.22 (0.08 - 0.57)

CI: confidence interval.

^a Test of fixed-effects least squares mean significantly different (t statistic = 2.27, df = 34, *P* = 0.030).

^b Test of fixed-effects least squares mean significantly different (t statistic = 3.09, df = 34, *P* = 0.004).

^c Test of fixed-effects least squares mean significantly different (t statistic = 3.08, df = 34, *P* = 0.004).

A comparison between observed and expected core and home range areas is shown in Table 10.

Table 10: Observed and geometric least squares means (Geo-LSM) of core and home range areas (ha) for rabbits radio-tracked between autumn 1999 and winter 2001. Core and home ranges have been described using kernel density and mean convex polygon (MCP) methods

Area	Sex	n	kernel 50%		kernel 95%		MCP	
			Mean	Geo-LSM	Mean	Geo-LSM	Mean	Geo-LSM
Core range:								
	Male	24	0.51	0.20	3.07	1.11	1.02	0.36
	Female	21	0.21	0.16	1.19	0.92	0.43	0.30
Total		45	0.39	0.16	2.35	0.92	0.79	0.30
Home range:								
	Male	53	0.71	0.26	3.48	1.54	1.25	0.50
	Female	38	0.20	0.12	1.18	0.72	0.55	0.28
Total		91	0.50	0.19	2.52	1.13	0.95	0.39

Core range habitat

A breakdown of the habitat composition, summarised across all core areas is shown in Table 11. In 392 of 632 (61%) tracking locations with recorded habitat, natural living vegetation was used as a denning site and dead vegetation (referred to as scrub) was the habitat for 118 of 632 (19%) tracking locations. In 15% of tracking locations, rabbits were found grazing during daytime and in 5% of cases, burrows were used.

Table 11: Vegetation types used by rabbits as denning sites located during daytime

Vegetation type	Number (%)
Gorse (<i>Ulex europaeus</i>)	276 (44%)
Broom (<i>Cytisus scoparius</i> L.)	86 (13%)
Toi toi (<i>Cortaderia</i> sp.)	25 (4%)
Scrub ^a	118 (19%)
Pasture	97 (15%)
Burrow	30 (5%)

^a Mainly dead vegetative material.

Death location in relation to home range centroid

Descriptive statistics of the distance (m) between the estimated home range centroid and death location are shown in Table 12. Deaths from RHD occurred closer to the home range centroid than deaths due to predation (LSD: $P = 0.014$) or other natural causes (LSD: $P = 0.074$).

Table 12: Distance between home range centroid and death location for different causes of death in wild rabbits, stratified for sex

Cause of death	Sex	<i>n</i>	Distance (m)		
			Mean (SD)	Median	Q1, Q3
Predation	Male	12	135 (122)	80	46, 241
	Female	7	67 (34)	71	29, 105
RHD	Male	6	54 (46)	51	14, 88
	Female	5	41 (39)	28	19, 71
Other deaths	Male	4	94 (56)	91	42, 150
	Female	2	64 (5)	64	61, 68

Q1: 25th percentile, Q3: 75th percentile.

Spatio-temporal clustering of RHD deaths

The most likely spatio-temporal cluster of RHD deaths was 160 metres in diameter, centred in the south-eastern quadrant of the study area and extending over a nine week period from 15 December 2000 to 20 February 2001 (log likelihood ratio statistic 8.64; $P = 0.054$). The home range area of rabbit 4899 (the first animal identified as RHD-positive in 2001) clearly extended into this region of the study area in 2001 (Figure 13).

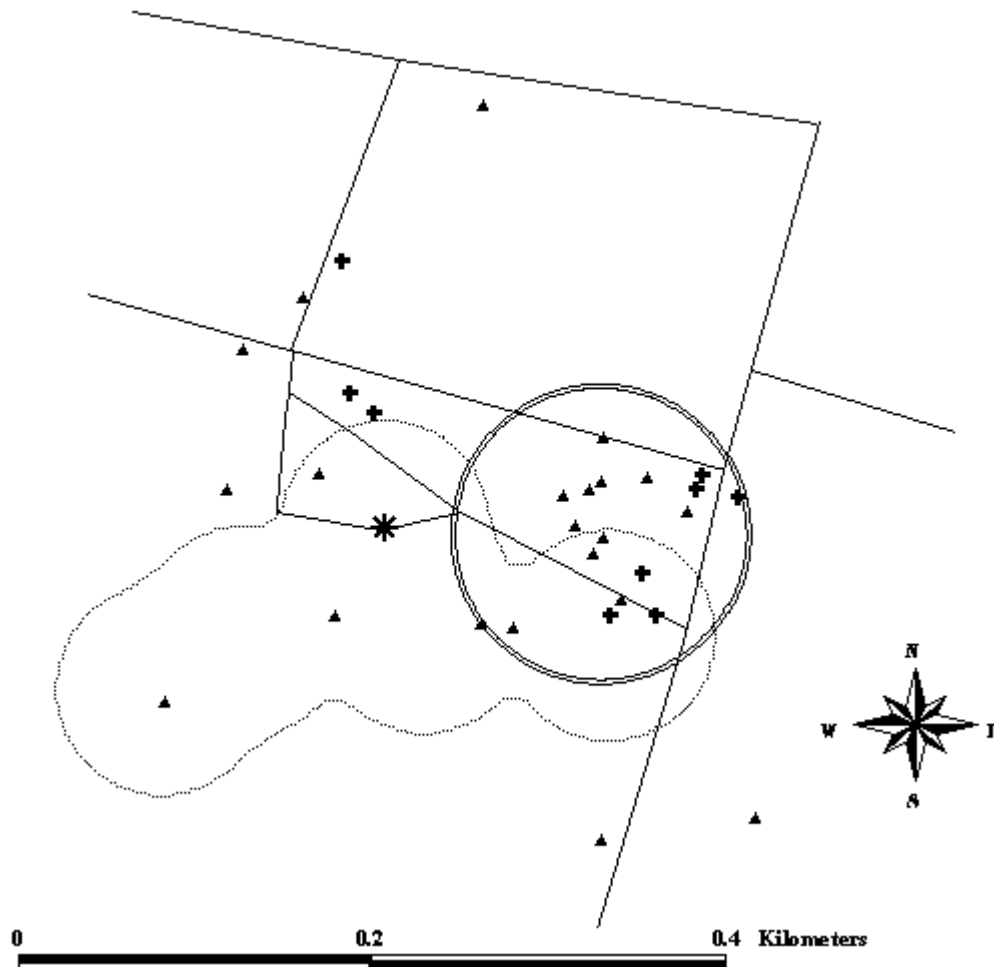


Figure 13: Death locations of rabbits dying between 1/03/1999 and 31/07/2001. Single solid lines represent fence lines; * are locations of deaths due to causes other than RHD in 2001; ⊙ are RHD death locations in 2001. The double solid line circle represents a space-temporal cluster of rabbit deaths (Relative Risk = 8.64, $P = 0.054$); ⊙ represents the first trapping location of rabbit 4899 and the dotted contour represents the home range area of rabbit 4899 in summer 2001 (kernel density estimate, 95% isopleth).

Discussion

Cause-specific mortality

Consistent with previous studies, predation was the predominant cause of rabbit mortality in this population. Studies on rabbit predation are difficult to conduct because rabbits are a preferred prey for many predator species and are usually consumed within minutes with any remains being subsequently scavenged by a variety of species.

Therefore uncertainty about the fate of some animals is inevitable. An experimental mortality study on post-emerged kittens in the Australian Capital Territory (south eastern Australia) by Richardson and Wood (1982) classified 56% of rabbits with unknown fate as victims of predation while a study of adult rabbit mortality in New South Wales, Australia was only able to definitively record the fate of 98 of 274 (36%) radio-tagged animals (Moriarty *et al.* 2000). Intensive monitoring in our study yielded a relatively high rate of carcass retrieval and diagnosis of death – especially during the 2001 RHD outbreak when all cadavers were retrieved fully intact. Only three confirmed RHD deaths were detected in 1999, but this is probably underestimated as some carcasses may have been completely devoured by predators. Misclassification as predation could have occurred if animals were weakened due to disease and became easy prey, or animals that had died from disease were subsequently scavenged.

Juvenile rabbits have a particularly high rate of mortality. Reddiex *et al.* (2002) attributed all deaths within a group of post-emergent, radio-collared rabbits to predation or RHD, but were not able to measure the importance of the respective causes, because rabbit carcasses had been scavenged heavily. Robson (1993), in a study of rabbit mortality in North Canterbury, New Zealand, showed that at least 38% of juvenile litters in breeding stops had been preyed upon, 29% of deaths were attributed to drowning, 20% of deaths were due to a combination of predation and drowning, and only 13% of litters were weaned successfully. Ground predators were responsible for more than half of the mortality of post-emergent kittens observed in the Australian Capital Territory study (Richardson and Wood 1982). Predation is also one of the major causes of death in adult rabbits. Of the 98 adult rabbit deaths recorded by Moriarty *et al.* (2000), 53 (44%) were thought to be due to predation.

In common with previous New Zealand studies, cats were the main predators at our site (Gibb *et al.* 1978). In comparison cats ranked third in importance as predators of adult rabbits (6% of all deaths) in a study in New South Wales, Australia, behind foxes (28%) and birds of prey (10%) (Moriarty *et al.* 2000). Cats are usually seen hunting rabbits by day and night (Gibb *et al.* 1978) and predation by cats occurred throughout the year at our site (Henning *et al.* 2003b). In contrast mustelids prey upon smaller rabbits than cats, and therefore their predatory activity is more dependent on the rabbit breeding season (Robson 1993). Unlike cats, ferrets and stoats dig into breeding stops to

kill where they mainly attack juveniles and nestlings (Gibb *et al.* 1978). Consistent with these observations, in our study area stoats and ferrets were only trapped in summer and autumn when abundance of younger rabbits was high (Henning *et al.* 2003a, 2003b). With regards to birds of prey Richardson and Wood (1982) reported a daily mortality risk of 0.7%, while rates of 4% were reported in an earlier Australian study (Calaby 1951). In New Zealand, the harrier is the only bird of prey, but its success in catching rabbits, especially full-grown animals, is poor (Gibb *et al.* 1978).

Deaths due to other natural causes (Table 7) and deaths due to RHD accounted for equivalent rates of mortality (20% and 19% respectively) but only RHD deaths were strongly clustered in time. Deaths during the 2001 RHD outbreak peaked within one week, similar to observations made at a study site in Central Otago, New Zealand, where RHD spread naturally (Parkes *et al.* 2002). Although no appreciable RHD epidemic was detected in 2000, RHDV was active on the site, possibly interacting or being replaced by other causes of mortality, in particular predation (Henning *et al.* 2003a). The overall mortality attributed to RHD in our study was 20%, which is similar to the proportion of RHD deaths (16%) observed in a Australian study by Moriarty *et al.* (2000). Pneumonia was the predominant cause of death we observed under the category of other natural causes and affected primarily young animals during the wet winter months, while *Eimeria* infection, often regarded as an important cause of death (Bull 1953; 1958), had only a minor influence on mortality. Similar observations were made in Australia, where parasitism by nematodes and coccidia were not important causes of death, implying that other mortality factors were responsible for density-dependent regulation in those populations (Hobbs *et al.* 1999).

In common with our study, most studies on adult mortality in males and females do not reveal gender specific differences (Parer 1977; Wood 1980; Gilbert *et al.* 1987; Webb 1993; Moriarty *et al.* 2000), although a higher mortality in males compared with females has been recorded (Cowan 1983).

Temporal patterns of mortality

Crude and cause-specific mortality rates are a function of age, which must be considered in the analysis. Kaplan-Meier estimates treat age as a continuous variable

with no grouping by age categories, and therefore no age-specific mortality rates were calculated. Predation resulted in death of many rabbits at a young age during the study period and young rabbits were also more vulnerable to other natural causes of death (e.g. pneumonia), while death from RHD occurred in older animals (after they had lost maternal antibodies and natural resilience).

Core and home ranges

The decision to only use rabbits with at least seven radio fixes per season resulted in many animals being excluded from core and home range analyses. Although this limited the power of the analysis, it did increase confidence in the accuracy of the core and home range estimates. The probability of the utilisation distribution was chosen to be 50% (the minimum area which contains 50% of the observations of an animal's core or home range) to represent the smallest size range estimate and 95% to include most of the area covered by an animal (Bond *et al.* 2002). Several authors have noted that an increase in the minimum area of probability (MAP) results in increased bias for non-parametric methods of range estimation (Worton 1989; Harris *et al.* 1990). Anderson (1982) recommended using a MAP of 50%, in particular for the core areas as they reflect areas of particularly high usage. It could be argued that the 95% isopleths may overestimate range areas, as they tend to be larger than minimum convex polygon (MCP) estimates, which are strongly influenced by outliers. Worton (1995) used brush rabbit (*Sylvilagus bachmani*) telemetry data to calculate 95% probability contours of non-parametric density estimators and regarded them as less biased than other estimation methods. Disadvantages of minimum convex polygons are that they can include areas never visited by the animal (Worton 1989). We used different analytical methods and were able to compare the estimates they delivered. Correlation between home range estimates from different methods was poor if a low number of radio-fixes was used per animal, but values of Pearson's correlation coefficient exceeded 0.9 if seven fixes or more were used as in our study.

Core range size

Core ranges are not often reported in the literature for rabbits, however recent research has tried to distinguish between core and home range areas (Bond *et al.* 2001;

White *et al.* 2003). Cowan (1983) described mean range sizes of 0.71 hectares for males and 0.43 hectares for females using maximum convex polygon and boundary strip estimation methods (Southwood 1966). Ranges of less than one hectare were also observed in Australian studies (Myers and Poole 1961). Daytime ranges in New Zealand of 0.25 hectares for males and 0.18 hectares for females have also been calculated (Gibb *et al.* 1978). Records in all these population studies were obtained during daytime or several hours before sunset and can be therefore regarded as core range estimates. The described core range estimates for the European rabbit were based on rabbit observation from lookouts, enabling only monitoring of animals within a distance of a few hundred metres and rabbits that were concealed by ground cover would not have been recorded. However, the use of radio-tracking allows recording of any animal movements and therefore larger estimates are to be expected. Using radio-tracking core ranges of 0.54 hectares for males and 0.57 hectares for female surface-dwelling rabbits were calculated, based on 60% isopleths using the harmonic mean method (White *et al.* 2003). Our geometric least squares mean core range sizes based on MCP (0.30 hectares) and 50% kernel (0.16 hectares) were similar to those reported studies, although our 95% kernel estimate was considerably larger (0.92 hectares). Although differences in core range areas across seasons were similar for each sex, estimated core range areas were larger for males than females in winter, when mating activity begins.

Home range size

Using a distance measure from the centre of activity for comparison (Hayne 1949), Gibb (1993) found a larger dispersion of males during daytime in summer and winter and reported that females were less variable in their seasonality. Based on kernel density estimation methods (95% isopleth), we detected significant differences in home range size between sexes during the winter and spring months, when males increase their movements towards does. This is consistent with observations that female rabbits tend to be more sedentary (Dunsmore 1974; Kunkele and von Holst 1996). Although gender-season differences were not readily observed using kernel 50% and MCP methods, home ranges showed clear differences between sexes. This indicates that feeding, territorial and mating behaviours have a different impact for the sexes. Parer and Fullager (1986) found that immature males disperse more frequently than other

classes of rabbits. Young adults are forced to move during the period of the greatest social pressure, when breeding groups are under formation, which accounts for the larger home range of male rabbits in autumn and the early winter months (Myers and Poole 1961).

Myers and Poole (1961) described a marked seasonal decrease in home range size during the breeding season, when activities related to territorial behaviour are at their maximum, and a marked expansion in the home range when breeding ceased. Breeding peaks on our sites were observed in spring and early summer (Henning *et al.* 2003a) and the smallest home ranges were also found in summer (males) and spring (females).

Home range size is influenced by habitat and distribution of available food (Gibb 1993; Zollner *et al.* 2000). However, variance component analysis revealed up to 50% variation between individual animals. The geometric least squares mean estimates were smaller than the observed values (but similar to the observed median values) and this discrepancy between estimated and observed values is possibly due to the large variation between animals. Similarly, observed means reported in wildlife studies may present a distorted picture of the actual area used by animals, as they are strongly influenced by excursions of single animals. High individual variability has been also reported from other telemetry studies (Gibb 1993; Hulbert *et al.* 1996). Rabbit home ranges recorded by radio-telemetry in the Orongorongo valley, New Zealand, had a minimum size of 2.6 hectares using the minimum convex polygon method, with the largest ranges for bucks being up to 6.5 hectares and for does about 4.5 hectares (Gibb 1993). These results suggest that geometric least squares means could be more appropriate than range estimates. In order to reveal differences in core and home range estimates for variables of interest, further range studies will require a larger number of animals to be monitored.

Core range habitat

Vegetation types were only compared for core areas since most tracking events were recorded during day-time. Spatial patterns of home ranges are dependent on the habitat. Dry conditions and loose ground allow burrowing and the development of large warren systems in Australia (Myers *et al.* 1994). However in different habitats the situation is

reversed, with one Australian study showing that up to 93% of radio locations were recorded in dense native vegetation, and 76% above the ground, when pastureland was surrounded by large areas of uncleared undergrowth (Wheeler *et al.* 1981). There are several reasons why rabbits den above ground. Australian studies suggested that surface dwelling could be the result of social regulation mechanisms, with socially sub-dominant animals being unable to establish themselves in warren systems (Mykytowycz and Gambale 1965; White *et al.* 2003). However, this could not have applied to our site, as warren systems were not present. Rabbits may be forced to den above ground if the ground material is inappropriate for burrowing (Gibb 1993), or choose to den above the surface in areas where sufficient and suitable ground cover is available, despite the soil being appropriate for burrowing (King *et al.* 1984). Ample ground cover was probably the reason why burrows were rarely used in our study. Rabbits prefer medium-height (50-100 cm) ground layer vegetation (Fa *et al.* 1999). On our site, vegetation grows up to 300 cm and very dense vegetation types such as gorse, broom and toi-toi grass were used as preferred denning areas. Dense vegetation provides shelter from weather conditions for many species (Williams and Karl 2002) and protects rabbits from predators and in particular birds of prey (Gibb 1993). The vegetation type might also have implications in the epidemiology of RHD. In areas with dense vegetation (and less burrowing) the lack of close contact and lower incidence of urinary and faecal contaminated burrows could limit the spread of RHDV infection. This would explain the lower incidence of RHD in higher rainfall areas where there is usually more vegetation available as ground cover. However, a positive correlation between RHD prevalence and vegetation cover has been described in an early report of the disease in Europe (Mitro and Krauss 1993). Moriarty *et al.* (2000) described that more surface- than warren-dwelling rabbits were killed by RHD. Contacts between surface- and warren-dwelling rabbits tend to be more aggressive than encounters between neighbouring warren dwellers and could therefore play a role in the long distance transmission of RHDV (White *et al.* 2003). In addition, if deaths occur under cover, carcasses may not be easily accessible for scavengers, which would facilitate access to carcasses by insects that can transmit RHDV mechanically (Henning *et al.* 2003c). The fly species *Oxysarcodexia varia* (Walker), considered as a main vector of RHDV (Barratt *et al.* 1998), was also the most abundant fly species on open pasture in this study (Henning *et al.* 2003c).

Death location in relation to home range centroid

There was evidence that RHD-infected rabbits died closer to their den site than animals dying from other causes. Short distances between an animal's home range centroid and death location are typical for acute diseases like RHD, where animals die suddenly. Carcasses close to den sites are more likely to be a source of new infection when infested by flies, than animals dying further away which are more likely to be rapidly scavenged. Deaths due to predation usually occur during feeding excursions on pasture, when rabbits are observed and killed by cats, ferrets or hawks.

Spatio-temporal clustering of RHD deaths

The spatial pattern of cause-specific mortality (Figure 13) illustrates that in this study RHD deaths were concentrated in small areas, whereas deaths due to predation or other natural causes were more widely dispersed. Environmental issues such as vegetation probably define these pockets of RHD deaths. The space-time cluster detected in this study was an area of small gorse bushes between grass patches, while most of the other parts of the study area contained extensive ground cover over many acres of open pasture. This half-open vegetation type with a scattered ground cover edging pastureland may reflect the best habitat for fly species transmitting the disease and could explain some of the observed patchiness of RHD within a small area.

The importance of carrier rabbits has been described previously by other authors (Cooke *et al.* 2000; Henning *et al.* 2003a). Circumstantial evidence indicates that a newly sero-converted animal (4899) in the population was the most likely index case for the 2001 RHD epidemic. We base this assertion on the following observations:

- i) There was no rabbit, which was sero-positive at a high diagnostic test dilution, trapped during the three weeks prior to the outbreak. High titres (>1:640) of antibodies are usually found in survivors of RHD outbreaks (Cooke *et al.* 2000). This individual probably had recovered from a previous infection as indicated by high IgG (1:640) and IgA titres (1:160) (Cooke *et al.* 2000; Henning *et al.* 2003a).

- ii) This rabbit was an adult male (6.4 months old), which migrated into the population, as he had not been trapped in the area earlier.
- iii) On the day of the first trapping of this animal, the first RHD death was observed. Eleven animals died during this outbreak (40% of the monitored population), but rabbit 4899 survived until predated on by a cat three months later.
- iv) The home range area of rabbit 4899 (Figure 13) clearly extended into the area of the RHD cluster in 2001.

RHDV can be transmitted by direct contact or through urine and faeces from infected animals (Ohlinger *et al.* 1993), but it has been shown that excreted RHDV can only survive for a limited time in the environment (Gregg *et al.* 1991; Henning *et al.* 2003d). Carrier rabbits can be the source of localised spread of the disease, aided by migration due to mating, feeding and territorial behaviour. Henning *et al.* (2003a) showed that immigration is a major factor influencing population immunity by increasing the number of susceptibles, but here it was also shown that immigration of infectious animals could induce new outbreaks.

There are always limitations when sparse rabbit populations are monitored. Gibb (1993) mentioned the importance of studies of low-density populations, as they may have different characteristics than higher density populations. He monitored only 60 rabbits for about 10 years, but drew important conclusions about social behaviour and space use of rabbits over time. Radio-collaring of every animal trapped and intensive monitoring in our study enabled us to describe a sparse population in detail in terms of temporal-spatial aspects of different causes of death.

This study has i) identified cause-specific mortality rates with predation being the major factor of rabbit mortality, ii) produced precise core and home range estimates by adjusting for seasonal and gender influences and repeated measurements of the same animal and iii) confirmed that RHD deaths appear clustered in time and space and close to rabbit denning sites. These findings have important implications for rabbit control with RHDV contaminated baits. The impact of the disease could be potentially optimised by distributing baits near burrow entrances or, where rabbits are mainly

surface dwelling, spreading baits in close proximity to ground cover used as denning sites.

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Stoat trapped on the study site



Hedgehogs captured within one day in spring

Chapter 4

Temporal relationships between seropositivity to *Rabbit haemorrhagic disease virus* in rabbits and other mammals in New Zealand*

*Submitted by J. Henning, P. R. Davies and J. Meers to *Wildlife Research*

Abstract

As part of a longitudinal study of the epidemiology of *Rabbit haemorrhagic disease virus* (RHDV) in New Zealand, serum samples were obtained from trapped feral animals that may have consumed rabbit carcasses (non-target species). During a 21 month period when RHDV infection was monitored in a defined wild rabbit population, 16 feral cats (*Felis catus*), 11 stoats (*Mustela erminea* L.), 4 ferrets (*Mustela furo*) and 126 hedgehogs (*Erinaceus europaeus*) were incidentally captured in the rabbit traps. The proportions of samples that were seropositive to RHDV were 6 of 16 (38%) cats, 2 of 11 stoats (18%), 1 of 4 ferrets (25%) and 5 of 126 hedgehogs (4%). Seropositive non-target species were trapped in April 2000, in the absence of an overt epidemic of rabbit haemorrhagic disease (RHD) in the rabbit population, but evidence of recent infection in rabbits was shown. Seropositive non-target species were found up to two and a half months before and one month after this RHDV activity in wild rabbits was detected. Seropositive predators were also trapped on the site between one and four and a half months after a dramatic RHD epidemic in February 2001. This study has shown that high antibody titres can be found in non-target species when there are no longer rabbits succumbing to RHD, although a temporal relationship was not detected statistically due to the small sample size. Predators and scavengers contribute to localised spread of RHDV through their movements.

Introduction

Rabbit haemorrhagic disease virus (RHDV) is a calicivirus that causes a peracute and often fatal disease in the European rabbit (*Oryctolagus cuniculus*). Although disease caused by RHDV, rabbit haemorrhagic disease (RHD), has not been reported in other species, the host range of the virus is not well defined and experimental infections of hares (*Lepus europaeus*) have yielded conflicting results (Ohlinger *et al.* 1990; Mocsari 1990; Löliger 1990; Kölbl *et al.* 1990; di Modugno and Nasti 1990; Du 1990; Chasey *et al.* 1992). In countries where introduction of RHDV to control pest rabbit populations has been considered, a major concern has been the risk that non-target species, particularly native mammals, could be susceptible to the disease. Serological evidence for cross-infection of non-target species was evaluated in relation to the proposed introduction of RHDV to control wild rabbits in both Australia and New

Zealand (Lenghaus *et al.* 1994; Buddle *et al.* 1997; Gould *et al.* 1997). Although seroconversion occurred in mice (*Mus musculus*) and kiwis (*Apteryx australis*), no evidence of a productive viral infection was detected in either species (Lenghaus *et al.* 1994; Buddle *et al.* 1997). Infection of humans, despite high levels of RHDV exposure, has not been detected serologically in Australia or New Zealand (Carman *et al.* 1998; Carman 1999; Greenslade *et al.* 2001). However some researchers have alluded to the potential for mutation of RHDV that could enable it to cross species barriers and cause disease (Smith *et al.* 1998a; Smith 1999). Both predators and scavengers of rabbits are likely to be exposed to RHDV when the disease is prevalent in wild rabbit populations, and seroconversion to the agent has been demonstrated in foxes (*Vulpes vulpes*) fed RHDV infected liver (Leighton *et al.* 1995). Field studies have demonstrated RHDV antibodies in free-ranging foxes in Germany (Dedek and Frölich 1997; Frölich *et al.* 1998) and recently in several predatory and scavenging species in New Zealand (Parkes *et al.* 2003). However seropositivity in these studies was not investigated in relation to the occurrence of RHD in associated rabbit populations.

An intensive longitudinal study of RHD in a wild rabbit population in New Zealand provided an opportunity to compare temporal patterns of RHD infection of rabbits with the serological status of other feral mammals trapped at the study location. This paper is the last of three papers reporting the findings of these investigations (Henning *et al.* 2003a; 2003b).

Materials and methods

This study was conducted on a 30 hectare site in the Manawatu region of New Zealand (Henning *et al.* 2003a; 2003b). A rabbit-proof fence enclosed 2.5 hectares of the site, and traps were incorporated into this fence to capture animals moving from one side of the fence to the other. The live-capture traps were set two nights per week, and trapped rabbits were identified, examined and released. Animals that wanted to cross the fence-line had to pass via the traps, although traps could be bypassed by climbing animals such as cats. In the period from September 1999 until June 2001, any trapped non-target species were anaesthetised by intramuscular injection of ketamine hydrochloride (100 mg/ml; Phoenix Pharm Distributors Limited, Auckland, New Zealand) to enable examination and sample collection. For each animal, the body

length, sex, weight, maturity and body condition were recorded and a 1 ml blood sample collected from the heart. Serum samples were tested for RHDV antibodies with a competitive ELISA (Capucci *et al.* 1991) at dilutions 1:10, 1:40, 1:160 and 1:640. Samples were classified as positive if the percentage inhibition was larger than 50% at a 1:40 dilution (Parkes *et al.* 2002). Hedgehogs were released away from the study site, and predator species were euthanased by an intracardiac injection of sodium pentobarbitone (Pentobarb 300, 300 mg/ml; National Veterinary Supplies Limited, Auckland, New Zealand).

Rabbits trapped on the site were tested for RHDV antibodies with the competitive ELISA and also with isotype ELISAs for IgG, IgM and IgA as described previously (Henning *et al.* 2003a). Rabbit carcasses retrieved by radio-tracking (Henning *et al.* 2003b) were autopsied and tested for RHDV on liver tissue using the reverse transcriptase polymerase chain reaction (RT-PCR) (Nowotny *et al.* 1997). A small sample ($n = 8$) of non-target species was also screened for RHDV using RT-PCR.

Chi squared and Fisher's exact tests were used to compare count data, and odds ratios were calculated. Logistic regression was used to test for the influence of sex, reproductive status, body condition and weight on seropositivity to RHDV (Hosmer and Lemeshow 2000).

Results

Results for rabbits have been reported in detail elsewhere (Henning *et al.* 2003b). Briefly, high levels of IgM indicating recent RHDV infection were detected in rabbits in April 2000 and February 2001. RHDV was detected in liver tissue from dead rabbits in February 2001, but not in April 2000. Therefore, there was evidence of RHDV transmission in the rabbit population in the months of April 2000 and February 2001, although confirmed mortalities were limited to February 2001.

A total of 158 non-target animals was trapped and sampled over the study period, including 16 feral cats, *Felis catus* (10%), 11 stoats, *Mustela erminea* L. (7%), 4 ferrets, *Mustela furo* (3%) and 126 hedgehogs, *Erinaceus europaeus* (80%). Significantly more male hedgehogs ($P = 0.04$) were trapped than females. The apparent prevalence of

animals with antibodies to RHD varied between species, being highest in cats (38%), and lowest in hedgehogs (4%) (Table 13).

Table 13: RHDV sero-prevalence in non-target species stratified by sex

	males		females		seropositive overall
	seropositive (%)	<i>n</i>	seropositive (%)	<i>n</i>	
Cat	50	6	30	10	38
Stoat	17	6	20	5	18
Ferret	0	0	25	4	25
Hedgehog	3	75	6	52	4

All 4 ferrets, including the single seropositive ferret, were trapped in January 2001. Trapping of cats, stoats and hedgehogs occurred more evenly during the study period. The temporal patterns of detection of seropositive samples from these species is shown in Figures 14-17. Positive samples were collected from cats in October and December 1999, April 2000, and in March, April and June of 2001, from stoats in April and May 2000, and from hedgehogs in February 2000 and April 2001. Antibody titres higher than 1 in 40 were found in one cat trapped in December 1999 (1:160), one stoat trapped in May 2000 (1:160), one hedgehog trapped in April 2001 (1:640), and two cats trapped in March and April 2001 (1:640).

No statistically significant association between potential physiological risk factors and the odds of seropositivity was detected using multivariable logistic regression. Seropositive non-target species were found up to 2 weeks before and up to 15 weeks after the recovery of an RHDV infected rabbit carcass in 2001. In 2000, seropositive non-target species were found from 10 weeks before to 4 weeks after the detection of a recent infection in a rabbit. Although there was a trend that predators were more likely to become seropositive in the two months before or after the detection of RHDV in rabbits (Figures 14-17), this was not supported statistically. The odds ratio for predators being seropositive between two months prior and two months after RHDV evidence in rabbits was 1.67 (95% CI: 0.33-8.36) compared to all other times of the year. No RHDV was detected by RT-PCR on liver tissues of three seronegative animals (2 stoats, 1 ferret) and 5 seropositive animals, comprised of two cats (seropositive at 1:40 and 1:640), two stoats (seropositive at 1:40 and 1:160) and one ferret (seropositive at 1:40).

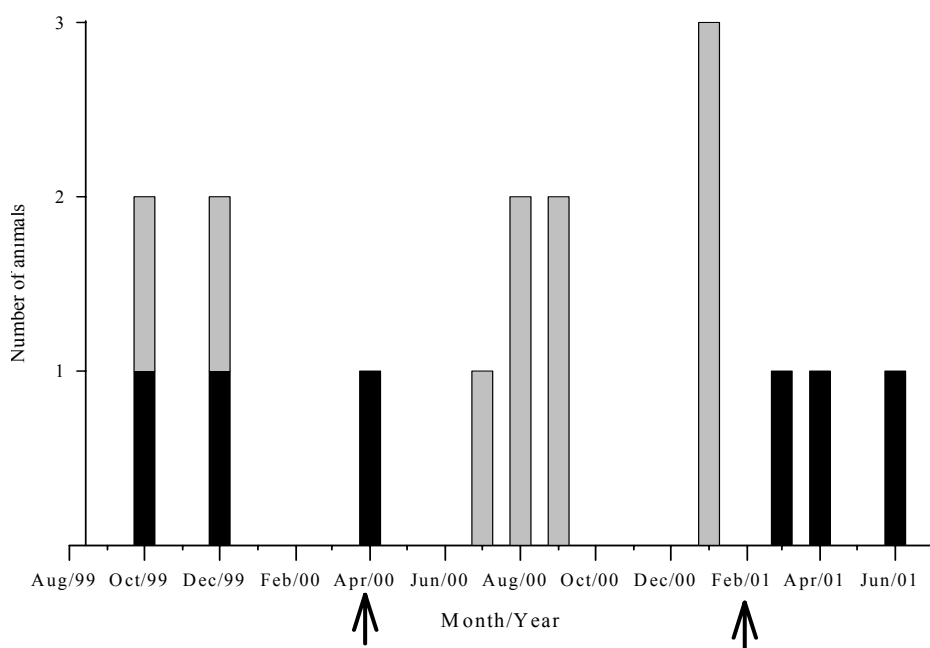


Figure 14: Number of RHDV seropositive (black) and seronegative (grey) cats trapped between August 1999 and June 2001. Arrows indicate months in which RHD transmission was observed in rabbits.

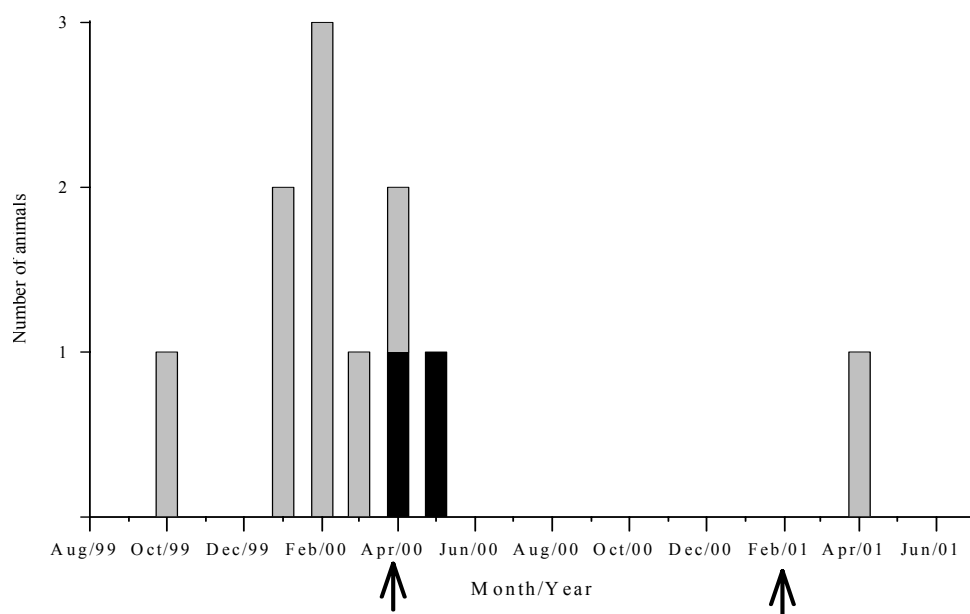


Figure 15: Number of RHDV seropositive (black) and seronegative (grey) stoats trapped between August 1999 and June 2001. Arrows indicate months during which RHDV transmission was observed in rabbits.

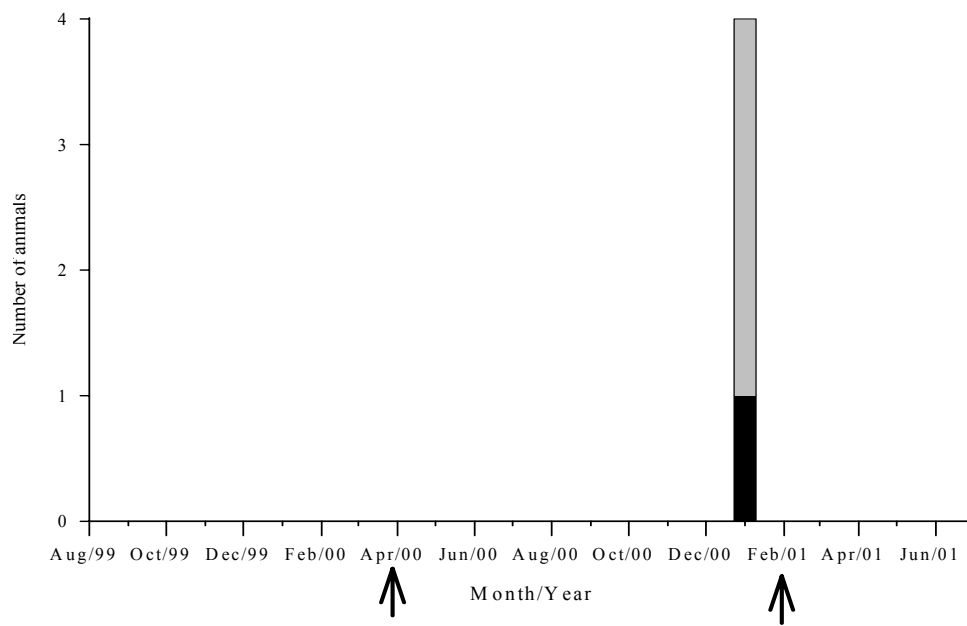


Figure 16: Number of RHDV seropositive (black) and seronegative (grey) ferrets trapped between August 1999 and June 2001. Arrows indicate months during which RHDV transmission was observed in rabbits.

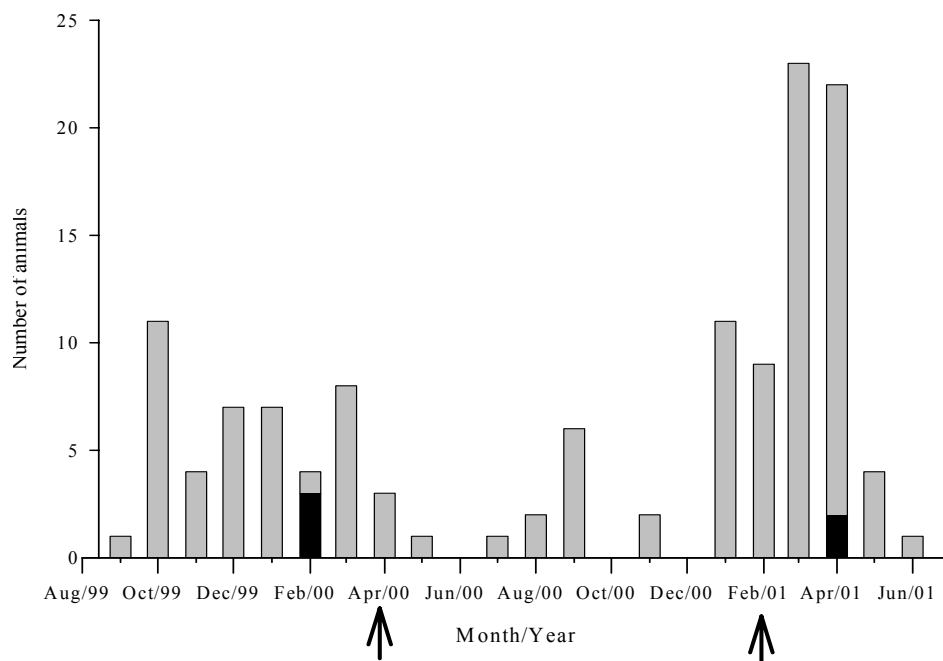


Figure 17: Number of RHDV seropositive (black) and seronegative (grey) hedgehogs trapped between August 1999 and June 2001. Arrows indicate months during which RHDV transmission was observed in rabbits.

Discussion

Interest in RHD infection of non-target species relates to concerns about i) detrimental ecological outcomes on non-target species that infection, viral replication and potential mutations of RHDV could have, and ii) the role that predatory and scavenging species may have in dissemination of the disease. Our observations complement a concurrent New Zealand report (Parkes *et al.* 2003) on antibody responses to RHDV in predators, scavengers, and hares. In the current study we were able to compare the time of RHD seropositivity detection in non-target species to events of RHD transmission in rabbits.

Rabbit haemorrhagic disease virus is generally considered to be highly species-specific (Ohlinger *et al.* 1993; Lenghaus *et al.* 2000; Cooke and Fenner 2002). However, this opinion is not universally accepted (Smith 1999), as there is evidence that some caliciviruses can infect and spread among multiple hosts (Smith and Boyt 1990). The best documented example of this is the San Miguel sea lion virus (SMSV). Several serotypes of SMSV having been isolated from a range of species of marine mammals and fish (Smith *et al.* 1980) and this virus can also cause vesicular disease in humans (Smith *et al.* 1998b) and in experimentally infected swine (Barlough *et al.* 1986a; Berry *et al.* 1990). Moreover, the agent of vesicular exanthema of swine, which causes clinical signs indistinguishable from foot and mouth disease, is thought to have emerged from SMSV (Barlough *et al.* 1986b; Smith *et al.* 1998a), and a Vesicular exanthema of swine-like calicivirus has recently been isolated from an aborted bovine foetus (Smith *et al.* 2002). In contrast, other caliciviruses, such as *Feline calicivirus* (FCV) and Norwalk calicivirus, appear to have a very limited host range (Wyatt *et al.* 1978; Seal *et al.* 1993; Lenghaus *et al.* 2000).

Similar clinical findings, morphological similarities, and geographic distributions between RHDV and the *European brown hare syndrome virus* (EBHSV) in European hares suggested that RHDV may have developed from EBHSV (Capucci *et al.* 1991; Cancellotti and Renzi 1991). However it is now known, that RHDV and EBHSV are two distinct caliciviruses (Capucci *et al.* 1991; Nowotny *et al.* 1997). The apparent diversity of biological behaviour (i.e. host specificity) among viruses classified as caliciviridae

means that it is questionable to base arguments about the risk of inter-species transmission of RHDV solely on analogy.

Due to resource limitations, in this study we routinely conducted clinical and serological evaluations of non-target species without the more definitive diagnostic procedures (i.e. histology and PCR). However, there was no evidence of clinical disease in any of the non-target species captured, and the limited PCR testing conducted was negative for RHDV. To date, there has been only one published report on recovery of RHDV from animals other than lagomorphs. Shien and Lee (2000) injected piglets subcutaneously with $10^{6.5}$ rabbit LD₅₀ of RHDV and found that virus was detectable by PCR in lung and liver up to 14 days post inoculation, and that the exposure led to low antibody titres of short duration.

New Zealand is ecologically unique in that, with the exception of two species of bats, it has no native terrestrial mammals, and almost all feral mammals are classified as pests. The most serious ecological concern in relation to RHD in New Zealand is the arguably unlikely potential for infection of non-mammalian hosts, most notably native bird species. Such events require both a mechanism of exposure to virus under field conditions and capability of the virus to infect the host. Probably a more realistic, although indirect risk of RHD to the New Zealand ecosystem is the exposure of native species to altered predator behaviour that could result from a rapid reduction of rabbit populations (Haselmayer and Jamieson 2001).

Four out of four North Island brown kiwis, *Apteryx australis matelli*, developed RHDV antibodies 14 days following intramuscular inoculation ($10^{6.5}$ rabbit LD₅₀). The birds had a rising titre over a period of 5 weeks and two birds remained seropositive until the end of the study at 136 days, by which time virus could not be demonstrated in tissues (Buddle *et al.* 1997). Similarly, seroconversion in the absence of detectable virus was observed following experimental infection of mice, and was considered most likely to be a response to injected antigen rather than an indication of viral replication (Lenghaus *et al.* 1994). With respect to species for which rabbits are an immediate food source, antibodies were detected up to 14 days after oral exposure of foxes to infected rabbit livers (Leighton *et al.* 1995). However, the study did not include tests for the presence of RHDV in fox tissues. Seroconversions have been also reported in domestic

dogs inoculated intraocularly and parenterally with RHDV (Simon *et al.* 1994). The seroconversions following experimental exposures to RHDV in mice, dogs and foxes do not emulate natural conditions (particularly parenteral routes) and thus does not constitute evidence for viral infection and replication. The latter is also the case with seropositivity in free-ranging animals, as described in this study. Seropositivity in free ranging animals allows identification of species that warrant experimental investigation as potential non-target hosts and reservoirs of the virus; these animals could possibly also serve as sentinels for RHDV.

We have previously shown that RHDV inactivated with ultraviolet light did not induce seroconversion in susceptible rabbits (Henning *et al.* 2003c). Therefore it is probable that consumption of inactivated RHDV by non-target species would also not induce a serological response. The possibility that viable RHDV consumed by non-target species may replicate in their tissues causing the observed seroconversion cannot be excluded.

Assuming rabbits are the sole significant reservoir of RHDV in an ecosystem, observed seroprevalence in non-target species will be determined by the performance characteristics (sensitivity and specificity) of the test, the prevalence of infected rabbits, and the nature and rates of interspecies contacts. RHDV epidemics are typically intermittent, occurring in annual or biannual cycles (Marchandeau *et al.* 2000; Calvete *et al.* 2002; Parkes *et al.* 2002) and at our study site only persisted for about 4 weeks (Henning *et al.* 2003a). The observed seroprevalence may be influenced by the population dynamics and incidence of capture of non-target species in relation to the temporal and spatial occurrence of these intermittent and transient rabbit epidemics. Despite a trend of seropositivity in non-target species being apparently associated with events of RHDV transmission in rabbits, our data were inadequate to support a temporal relationship statistically. Key limitations of the data were the small sample size, and heterogeneity over time in the populations of non-target species that were trapped (for example all 4 ferrets were trapped in a single month). The likely importance of the persistence of detectable titres to RHDV following exposure also needs to be acknowledged (Leighton *et al.* 1995). Unfortunately, the performance characteristics of serological tests for RHD in non-target species have not been systematically evaluated. A major concern in this respect is the potential for cross-reactions, particularly in

species such as cats, which are known to be susceptible to other calicivirus infections (Pedersen *et al.* 2000).

Given the existence of RHDV infected rabbits, the probability of seroconversion of predatory and scavenging species will depend on their feeding behaviours and the concentrations of virus in the consumed tissues. Feeding behaviours are likely to be influenced by season and the availability of other food sources. Seasonal scat examination of feral cats captured in a 10 year study of rabbits in the nearby Wairarapa region in New Zealand revealed that, at most times of the year, at least half the volume of faeces was composed of rabbit remains, and in some years this increased to 90 to 100% (Gibb *et al.* 1978). Other important food sources for cats, in decreasing order of importance, were birds, insects and mice (Gibb *et al.* 1978). Cats favour young rabbits as prey (Jones 1977; Borkenhagen 1978; Catling 1988) and rabbit consumption by cats is particularly predominant in spring, when many young rabbits are present (Gibb *et al.* 1969). Gibb and Fitzgerald (1998) observed an increase of young rabbits in the diet of cats in a period from 1984-1988 from 10 up to 50% in the spring and summer months, which is the breeding season of rabbits. Recent studies in New Zealand and Australia conducted after RHD became endemic and rabbit numbers declined, showed that rabbits remain the preferred prey of feral cats (Molsher *et al.* 1999; Gillies 2001).

Lagomorph remains were detected in 16% of stoat guts in an open forest valley in Fiordland, New Zealand, and were more prevalent (up to 26%) in years of low stoat density (Murphy and Dowding 1995). The fact that when small rodents are more abundant, they become a major prey item for stoats (Murphy and Dowding 1995), suggests stoats prefer to prey on smaller animals, including young rabbits. Ferret droppings examined from 1961 to 1964 showed variation with respect to content of remains from young and old rabbits (Gibb *et al.* 1978), with faeces containing twice as much remains of young rabbits compared with full grown rabbits in the breeding season. The overall proportion containing rabbit remains varied between 24% and 95%, depending on the abundance of rabbits, while other studies have found up to 65% of lagomorph remains in ferret stomachs (Smith *et al.* 1995).

Although birds were not captured in our study, the diet of harriers is similarly influenced by rabbit abundance and in particular the abundance of smaller rabbits (Gibb

et al. 1978). Gurr (1968) found 90% of harrier stomachs contained rabbit remains during periods of high rabbit abundance in Otago, while only 20% had traces of rabbit remains when abundance was low (Carroll 1968). In another study in the Mackenzie Basin, Pierce and Maloney (1989) found that rabbits constitute 80-99% of the harrier diet. It has also been reported that harriers had poor success rates in killing rabbits and mainly feed on rabbit carrion killed by other predators (Gibb *et al.* 1978). By analogy this would include rabbits infected with RHDV. With respect to environmental concerns about interspecies transmission of RHDV to native birds in New Zealand, a case could be made for the evaluation of RHDV infectivity in those avian species, such as harriers, that are most likely to be exposed to RHDV under natural conditions.

Mammalian predators also prey on other scavengers and predators. Cat faeces contained hedgehog and stoat remains, while hedgehog remains have been found in ferret faeces (Roser and Lavers 1976; Gibb *et al.* 1978). Secondary infection following consumption of infected non-target species is theoretically possible but unlikely. In cats the proportion of hedgehog and stoat remains did not exceed 6% or 1% respectively (Gibb *et al.* 1978) and in ferrets the proportion of hedgehog remains was only 8% (Roser and Lavers 1976). A study on pastoral farmland in New Zealand revealed scavenging of ferrets on ferret carcasses and hedgehogs scavenging on possum carcasses, but not on rabbit carcasses (Ragg *et al.* 2000). Although it was mentioned that this needs more exploration, these findings are consistent with our observations of a relatively low seroprevalence of RHDV in hedgehogs.

In our study, seropositive non-target species were trapped several weeks before and after RHDV emergence in rabbits, suggesting that they either moved in from neighbouring RHDV infected areas into the study region, or that they had contact with infected rabbit carcasses not detected on our site (Henning *et al.* 2003b). Prolonged high antibody titres in non-target species may be the consequence of their consumption of large amounts of rabbits that died from RHD or through them consistently preying on seropositive rabbits, which survived previous RHD outbreaks and contained RHDV in their tissues. The latter seems less likely, as RHDV could not be detected in liver tissue of rabbits shot in a cross-sectional survey, despite the rabbits having high RHDV antibody serum titres (Henning and Davies 2003).

Scavengers and predators may play a role in the localised spread of RHDV via environmental contamination by saliva, fur or feet. In addition, studies on domestic dogs fed infected rabbit livers showed that dogs shed virus infective for rabbits (Simon *et al.* 1994) and it appears likely that the transient faecal excretion of viable virus following consumption of large doses of RHDV is another probable mechanism for the localised spread of RHDV. Interaction between predation and RHD have been reported previously (Reddiex *et al.* 2002) and this could be due to the localised spread of RHDV through non-target species.

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Fly bottle trap (modified Western Australian style) after one week of trapping in summer



Oxysarcodexia varia Walker

Chapter 5

Influence of weather conditions on fly abundance and its implications for transmission of *Rabbit haemorrhagic disease virus* in the North Island of New Zealand*

*Submitted by J. Henning, F. R. Schnitzler, D. U. Pfeiffer and P. R. Davies, to *Medical and Veterinary Entomology*

Abstract

Blowflies (Diptera: Calliphoridae) and flesh flies (Diptera: Sarcophagidae) are potential vectors of *Rabbit haemorrhagic disease virus* (RHDV) in New Zealand. The associations between habitat and weather factors on the abundance of these flies were investigated. Between October 1999 and June 2001, flies were trapped on open pasture and in dense vegetation patches on farmland in the Himatangi area of the North Island. Five calliphorid species were trapped commonly at scrub edges and the most abundant sarcophagid, *Oxysarcodexia varia* Walker, was trapped mainly on open pasture. Overall abundance of flies varied according to habitat and species, and species numbers differed between seasons and years. The all-day minimum temperature three weeks before trapping was a significant variable in all models of fly abundance, while average rainfall did not affect fly abundance. The all-day temperature range was significant only for *O. varia*. The influence of other climatic factors varied between fly species. Climate dependent variations in fly abundance may contribute to the risk of transmission of rabbit haemorrhagic disease, which occurred intermittently on the site during the study period.

Introduction

Flies can affect animal health and production through irritating animals and interfering with grazing or eating behaviour, or by acting as biological or mechanical vectors of pathogens (Greenberg, 1973; Wardhaugh & Morton, 1990; Hall & Wall, 1995; Heath & Bishop, 1995; Kettle, 1995; Fischer *et al.*, 2001; Heath, 2002).

Rabbit haemorrhagic disease virus (RHDV) was introduced illegally into New Zealand in 1997 (Thompson & Clark, 1997). The virus causes peracute disease and high rates of mortality in rabbits, and is used as a biological control tool for wild rabbit populations (Sanson *et al.*, 2000; Parkes *et al.*, 2002). The effectiveness of the disease in reducing pest rabbit populations appears to be highly variable, and epidemics generally occur intermittently at annual or bi-annual intervals (Parkes *et al.*, 2002; Henning *et al.*, 2003a). The abundance and activity of insect vectors may be one factor that contributes to the observed variability in the impact of RHD (Barratt *et al.*, 2001). Climatic factors are major determinants of fly abundance and population dynamics (Davies & Hobson, 1935; Evans, 1936; Davidson, 1944; Hughes & Sands, 1979; Vogt *et al.*, 1983, 1985;

Vogt, 1986, 1988; Wall *et al.*, 2001) with different fly species preferring different habitats (Dymock & Forgie, 1993; Gleeson & Heath, 1997; Heath & Appleton, 2000). In New Zealand, eight fly species have been identified as potential vectors of RHDV (Crosby & McLennan, 1996) and their role was investigated in field studies (Barratt *et al.*, 1998; Heath *et al.*, 1999).

The aims of this paper are: (1) to describe the seasonal abundance of the most common blowfly and flesh fly species likely to be involved in RHDV transmission in the North Island of New Zealand, (2) to identify the influence of habitat and season on the abundance of fly species, and (3) to investigate their association with climatic variables.

Materials and Methods

Study site

The study site was located near Himatangi (longitude 175.317, latitude -40.400), in the North Island of New Zealand. The vegetation on the study site consisted predominantly of grass patches, dense gorse bush (*Ulex europaeus*) and scrub. Twelve traps were placed in two lines 50 m apart with 50 m intervals between traps on the same line (Figure 18). Six traps were placed on open pasture, while the other six traps were located within or on the edge of dense gorse patches.

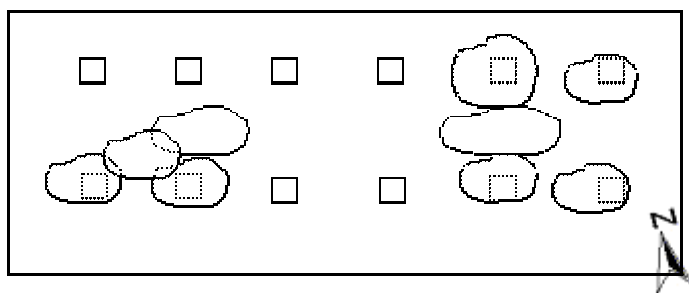


Figure 18: Positions of flytraps located under shrubs (□) and on open pasture (□) in relation to vegetation.

Sampling and identification of flies

A modified Western Australian (WA) fly trap (Cole *et al.*, 1993) supplied by Landcare Research (PO Box 282, Alexandra, New Zealand) was used for the collection of flies. The design varied from the WA trap in that the bait chamber, which consisted of a lowly positioned soft drink bottle, was replaced with a clear plastic screw top container. The funnel and the modified screw top lid were glued to the base of the collecting chamber. Each trap was baited with 150 g of 15 mm slices of bovine liver (Cole, 1996). Sodium sulphide (5%) was poured over the bait to ensure preservation of the bait and to act as an additional fly attractant (Fuller, 1934).

Traps were set at fortnightly intervals from October 1999 to September 2000 and from January 2001 to June 2001. Flies were sampled for one week and then collected from traps. Flies were anaesthetised in the field by carbon dioxide infusion and then collected in screw top containers and killed and stored at -80°C before further processing. Flies were transferred into 70% ethanol and were identified and counted using a stereo-microscope (Nikon SMZ-1B, 10x21).

Climate data

Squirrel data loggers (ELTEK Ltd, Cambridge, UK) were placed on open pasture and between gorse bushes to record temperature and relative humidity at two-minute intervals. The Squirrel data logger software SQREM version 2.31 (ELTEK Ltd, Cambridge, UK) was used to transfer the data to a laptop computer. The climate data were summarised into hourly averages and 28 climate variables were calculated (Appendix 1). These included the average, minimum and maximum temperature and range, and the minimum humidity and range. The maximum humidity range was not used for analysis as little variation occurred for this variable. All climate variables were created for all-day (24 h), day-time (6 am-6 pm) and night-time (6 pm-6 am) periods. These were converted to weekly averages because fly trapping was conducted over one week periods. Weekly means of daily sunshine were calculated using data from the Foxton weather station (longitude 175.300, latitude -40.467) and weekly means of hourly wind and rainfall data calculated using data from the Levin weather station (longitude 175.275, latitude -40.633).

Data analysis

Analyses were conducted using the statistical software packages SPSS 11.0 (SPSS Inc, Chicago, IL, USA) and STATA 7.0 (STATA Corporation, College Station, TX, USA).

The total number of flies per species and the number of flies per species across different trap vegetation environments were compared using Chi squared tests. A random effect Poisson regression model was used to compare fly abundance in different trap vegetation environments (pasture and gorse) per month (Cameron & Trivedi, 1998). The calendar month for each year and the trap vegetation environments were used as fixed effects and the fly species was incorporated as a random effect in the model.

Two approaches were used to reduce the number of climate variables in the analysis.

(1) A factor analysis approach was used to identify relationships present in the original variables. Factors created reflected unique variance separated from the common variance of these climate variables. Eigenvectors were selected using the latent root criterion and scree plots (McGarigal *et al.*, 2000), and were rotated (varimax rotation) to simplify their interpretation (Hair *et al.*, 1998). Rotated eigenvectors were used to identify single climate representative variables, which were entered into the regression models.

(2) Testing for multicollinearity was based on inspection of correlation matrices between all climate variables. A Pearson correlation greater than 0.7 was used to identify a high correlation between two variables and suggested the presence of multicollinearity.

Fly count data were transformed using $\ln+1$ to normalise their distribution. A univariate GLM model was developed with linear and exponential (where appropriate) climate variables as predictors. Lagged values of climate variables for up to three weeks before a particular week of trapping were also included as predictors to account for the climate dependent pre-adult phase in fly life cycles. The trap vegetation environment (pasture and gorse) was used as a categorical fixed effect variable and a stepwise forward selection procedure was used to select influential climate variables and interaction terms ($P < 0.05$). Comparisons between the full model and the best parsimonious model were conducted using the adjusted R squared statistic (Hair *et al.*, 1998) and regression diagnostics were used to assess the goodness-of-fit of the model.

All models were computed with and without trap number as a random effect to account for the repeated measure characteristics of the dataset, and the statistical significance of the random effect was tested using the Hausman specification test for random effects (Hausman, 1978). Separate regression analyses were conducted for each fly species. In addition, the abundance of *Oxysarcodexia varia* Walker was assessed in relation to habitat.

Results

A total of 29,415 flies were counted. Fly abundance was not distributed normally for each sampling occasion and abundance was different among individual species ($\chi^2 = 5885.75$, $P < 0.001$, $df = 5$) (Figure 19). For each species, the number of flies differed significantly between the two trap vegetation environments (pasture and gorse bushes) ($\chi^2 = 3245.92$, $P < 0.001$, $df = 5$) (Figure 20).

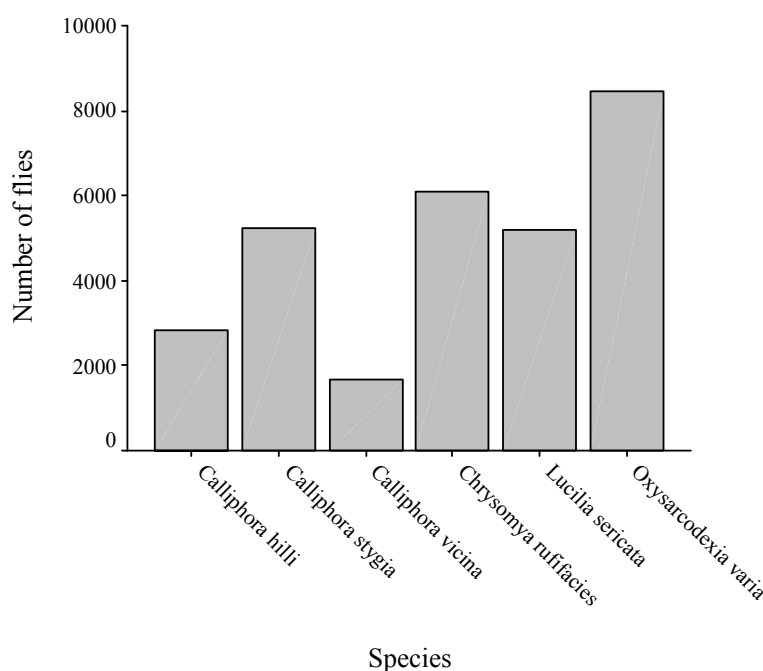


Figure 19: Number of flies trapped per species over the entire study period.

O. varia was the most abundant fly ($n = 8,436$), and was trapped mainly on open pasture, while all other blowflies were present in higher numbers on the gorse edges. Of the six species studied, the least abundant species was *Calliphora vicina* Robineau-Desvoidy ($n = 1,668$), followed by *C. hilli* Patton ($n = 2,825$), *Lucilia sericata* Meigen

($n = 5,173$), *C. stygia* Fabricius ($n = 5,220$) and *Chrysomya rufifacies* Macquart ($n = 6,093$).

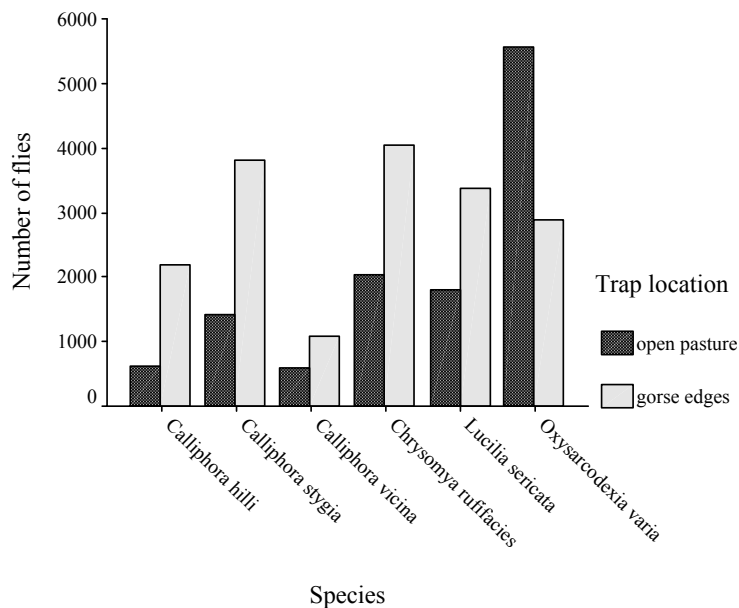


Figure 20: Number of flies trapped per species on open pasture and gorse edges.

The total number of flies of each species caught over the total study period is displayed in Figure 21.

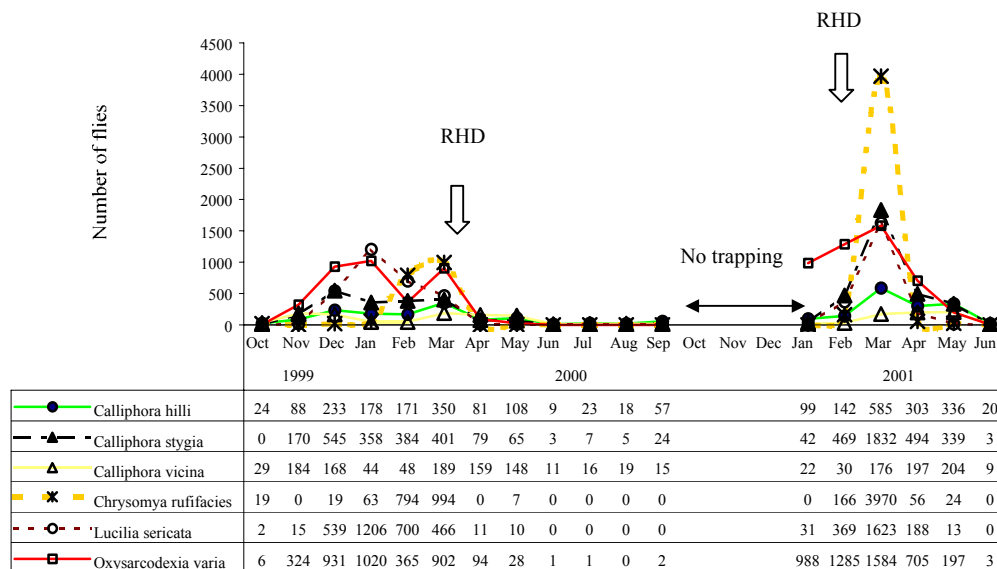


Figure 21: Abundance of blowfly and flesh fly species from October 1999 until June 2001 (excluding October 2000 to January 2001) at Himatangi.

Fly numbers were greatest in summer (Southern hemisphere) and decreased rapidly to very low numbers in winter, and varied between species and between years. *O. varia* numbers peaked in January 2000 and then declined by almost two-thirds in February

2000. They then returned to similar numbers as in January by March 2000 before decreasing to 10% of the peak value by April 2000. In the following year, abundance of *O. varia* was about 40% higher in summer (January-March) 2001, and peaked in March. In both years, *C. rufifacies* numbers peaked in March, but were approximately four fold higher in 2001 compared to 2000. *C. stygia* numbers were highest in December 1999 and remained at similar levels until March 2000. *C. stygia* numbers were still very low in January 2001, reached a peak in March 2001 and then remained elevated until May 2001, which was later than the same season in the previous year. *L. sericata* also had its highest abundance later in 2001 (March) than in the preceding year (January), but dropped dramatically in April for both years. No difference in the abundance peaks was observed for *C. hilli*. Abundance of this fly species peaked in March in both 2000 and 2001, but persisted at higher numbers for an additional two months in 2001 compared to 2000. *C. vicina* was highly abundant in the late summer and autumn period (March-May) of both years, but the additional spring peak in November-December 1999 was not observable in 2000 as no trapping occurred during this period. *C. hilli* and *C. vicina* were the predominant fly species over the winter months. A comparison of fly abundance in the two vegetation environments over the 18 months of trapping using the Poisson regression model with fly species as a random effect, showed highly significant differences ($P < 0.01$) for all months, except for June 2000 ($P = 0.057$), July 2000 ($P = 0.614$), June 2001 ($P = 0.013$) and July 2001 ($P = 0.064$).

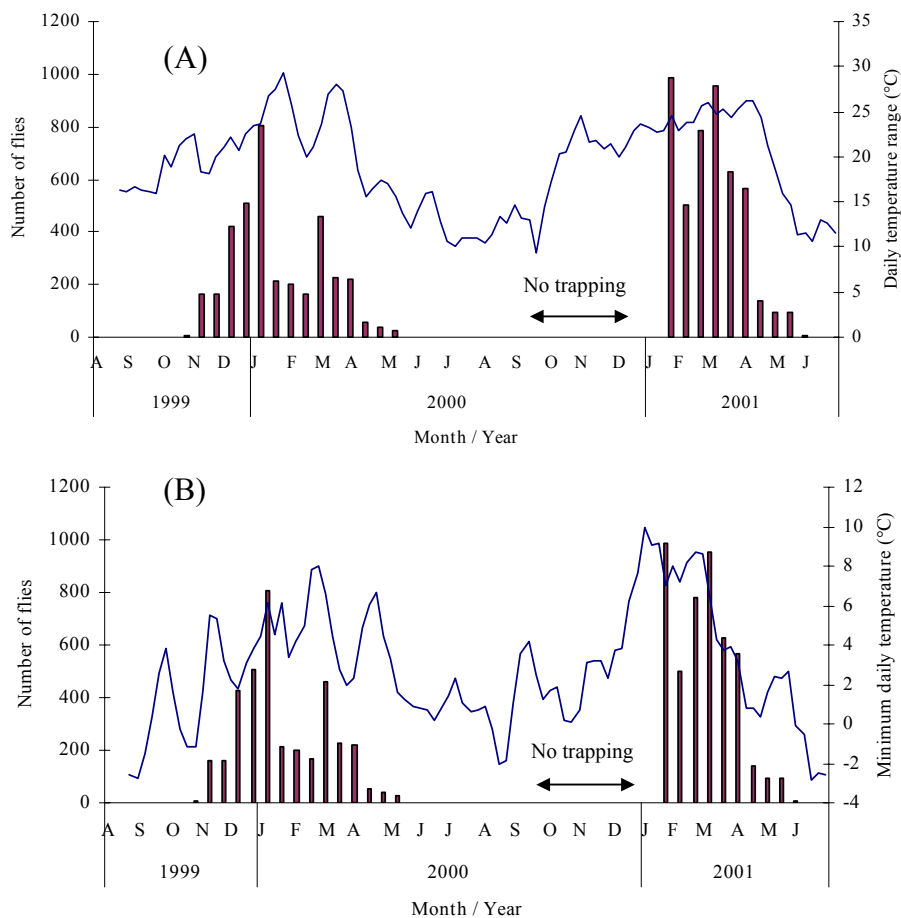
The factor analysis of all 28 climate variables revealed 5 factors with eigenvectors greater than 1, which together accounted for 94% of the total variation. After rotation, the 5 climate variables with the highest rotated factor loadings were selected (Table 14).

Table 14: Variables obtained from principal factor analysis and their number of correlations (correlation coefficient > 0.7) with other climate variables ($n = 28$)

Climate variable selected by factor analysis	Number of correlations with other climate variables
All day (24 h) temperature range	11
All day (24 h) temperature minimum	1
Average rain Levin	1
Night time (6 pm-6 am) humidity range	3
Average wind Levin	1

The correlation matrix revealed up to 13 correlations > 0.7 for a single climate variable. A list of variables selected by factor analysis and the number of correlations for each of these variables is also shown in Table 14. Four climate variables selected by factor analysis had fewer than 4 correlations higher than 0.7 with other climate variables, and one variable had a relatively high number of such correlations. Therefore we were confident, that the selected variables explained most of the variability in the climate variables, and that most of the multicollinearity was avoided by selecting these variables.

Figure 22 shows the climate pattern of these 5 variables selected by factor analysis for the most abundant fly, *O. varia*. Climate data are displayed by 3 weekly moving averages. The graphical pattern suggests that the maximum daily (24 h) temperature range, the minimum daily (24 h) temperature and the humidity range at night (6 pm–6 am) may influence the abundance of *O. varia*, while average daily rainfall and average daily wind speed do not show clear correlations with abundance for this species.



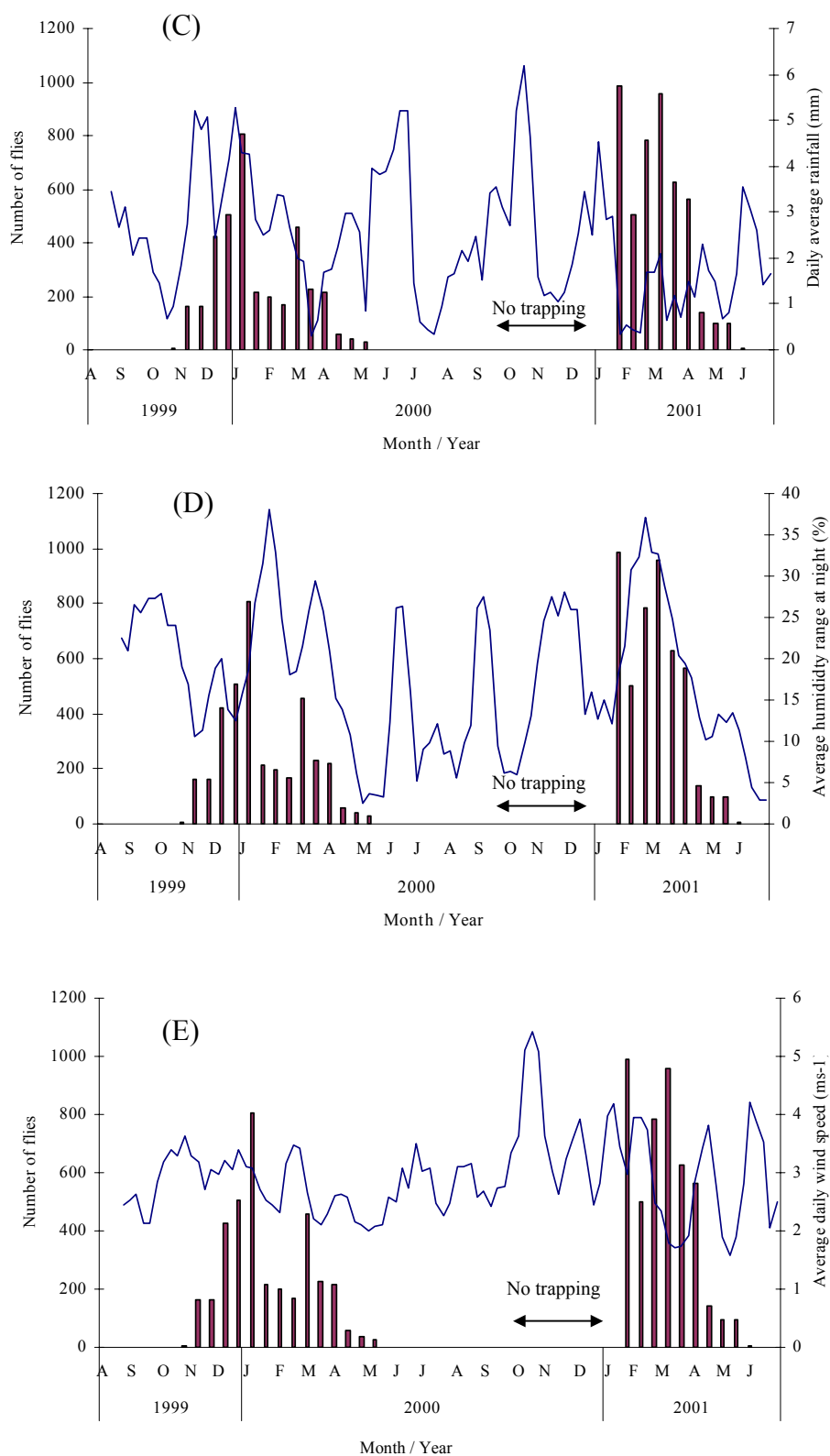


Figure 22 A-E: Climate pattern and abundance of *O. varia* from October 1999 until June 2001 (excluding *O. varia* abundance from October 2000 to January 2001) at Himatangi.

The GLM models for different fly species are shown in Table 15. The all day (24 h) minimum temperature three weeks before trapping was a significant variable in all models of fly abundance estimation. All day (24 h) minimum temperature for the week of trapping was significant for four species and the all day (24 h) temperature range was only significant for *O. varia*. The night-time (6 pm-6 am) humidity range three weeks before the trapping period was significant for three fly species. The abundance of two species was associated with the night-time (6 pm-6 am) humidity range in the week of trapping. Average wind speed was an important variable for three fly species. The categorical variable trap vegetation environment was significant for *C. hilli*, *C. stygia* and *C. vicina*. Average rainfall had no effect on any of the models.

Table 15: Summary of General Linear Models for climate factors influencing the abundance of six different fly species (log numbers +1).

	<i>Calliphora hilli</i>	<i>Calliphora stygia</i>	<i>Calliphora vicina</i>	<i>Chrysomya rufifacies</i>	<i>Lucilla sericata</i>	<i>Oxysarc. varia</i>
Variables	Coeff (s.e.)	Coeff (s.e.)	Coeff (s.e.)	Coeff (s.e.)	Coeff (s.e.)	Coeff (s.e.)
Intercept	1.51(0.24)	-0.16 (0.13)	1.53 (0.23)	0.12 (0.29)	-0.63 (0.10)	-1.87 (0.13)
AD T _{Rng}	-	-	-	-	-	0.15 (0.01)
AD T _{Min}	0.08 (0.02)	0.13 (0.02)	-	-	0.21 (0.02)	0.22 (0.02)
AD T _{Min} 3w lag	0.11 (0.02)	0.15 (0.02)	0.05 (0.01)	0.20 (0.02)	0.11 (0.02)	0.13 (0.02)
NT H _{Rng}	-	-	-	0.04 (0.01)	0.04 (0.01)	-
NT H _{Rng} 3w lag	-	0.04 (0.01)	0.03 (0.01)	0.03 (0.01)	-	-
Average wind	-0.28 (0.07)	-	-0.34 (0.07)	-0.43 (0.09)	-	-
Trap vegetation	-0.46(0.09)	-0.32 (0.13)	-0.34 (0.10)	-	-	-
Adjusted R²	0.40	0.45	0.19	0.50	0.55	0.68
AD	All day (24 h)		NT	Night time (6 pm-6 am)		
T _{Rng}	Temperature range		T _{Min}	Minimum temperature		
H _{Rng}	Humidity range		3w lag	3 weeks lag		
Coeff	Coefficient		s.e.	Standard error		

A comparison of GLM models for the two trap vegetation environments (pasture and gorse) for the most abundant fly, *O. varia*, is shown in Table 16. The all day (24 h) minimum temperature was significant in both models, while the all day (24 h) minimum

temperature three weeks before trapping was only significant for gorse trap catches. The variable all day (24 h) temperature range influenced only the fly abundance on pasture.

Table 16: Separate General Linear Model for the climate factors influencing the abundance of *O. varia* on pasture and gorse (log numbers +1).

	Pasture	Gorse	Overall
Variables	Coeff (s.e.)	Coeff (s.e.)	Coeff (s.e.)
Intercept	-2.39 (0.24)	-0.58 (0.15)	-1.87 (0.13)
AD T _{Rng}	0.20 (0.01)		0.15 (0.01)
AD T _{Min}	0.22 (0.02)	0.18 (0.03)	0.22 (0.02)
AD T _{Min} 3w lag		0.20 (0.03)	0.13 (0.02)
Adjusted R²	0.77	0.51	0.68
AD	All day (24h)	T _{Rng}	Temperature range
T _{Min}	Minimum temperature	3w lag	3 weeks lag
Coeff	Coefficient	s.e.	Standard error

Discussion

The data collected in this study allowed the abundance patterns of five Calliphoridae species and one Sarcophagidae species over a period of nearly two years to be determined. Peaks in abundance persisted for up to two months longer in the 2000/2001 summer/autumn season compared to the 1999/2000 season. Fly numbers for all species were generally higher in 2001 compared to the previous year. Abundance peaks of all species, except for *C. hilli* and *C. rufifacies*, appeared two months later in the 2000/2001 summer than in the previous season.

Cottam *et al.* (1998) observed several peaks of weekly-trapped blowflies per year in a similar geographical region and suggested that these peaks reflected emergence of successive fly generations. Our study also revealed multiple peaks for *C. hilli* (December 1999 and March 2000) and as well for *O. varia* (January and March 2000), but multiple peaks were not observed in the second season in 2001. The first peaks occurred earlier in the year (December 1999 for *C. hilli* and January 2001 for *O. varia*) compared to Cottam *et al.* (1998) who observed first abundance peaks in February/early March 1994, 1996 (trapping in this season started in February 1996) and 1997. This

suggests that the conditions influencing the biological activities of the flies differed between years. However, direct comparisons of weather patterns between only a few consecutive years are difficult because of the limited number of seasons.

Weather conditions affect all periods of the life cycle of a fly. Thus, fly population dynamics are always influenced by climatic conditions in the early part of the fly life cycle. Egg, larval and pupal survival depend on dung characteristics and soil conditions, which are strongly influenced by temperature and humidity (Hughes & Sands, 1979). Adult flies free-living in open air are also vulnerable to changes in weather conditions. Prolonged exposure to low temperatures increases adult fly mortality (Hughes & Sands, 1979) while rainfall (Cottam *et al.*, 1998), wind speed (Vogt *et al.*, 1983) and wind direction (Barratt *et al.*, 2001) all influence fly numbers.

Consecutive days with markedly different temperatures are more likely to reveal differences in response to temperature (Vogt *et al.*, 1985) than temperature ranges within a day. The use of weekly averaged data, as applied in our analysis, would have taken this into account. Based on life cycle data cited in the literature (Appendix 2), a three-week lag for climate variables was chosen to cover specific climatic conditions of the pre-adult phases of the trapped flies. Hence, the number of flies trapped in a specific week was defined as a function of the climate conditions in that week as well as the climate conditions three weeks earlier. Cottam *et al.* (1998) used two- and four-week lags in separate models to relate fly abundance to weather variables and concluded that weather recordings offset by two weeks did not account for variations in catch numbers. We decided that time lags of three weeks would be sufficient to explain climate variation of the pre-adult generation as the average fly life expectancy is between three to four weeks. By adding weather time lags to the coincident weather records we observed model improvements between 5 and 10%.

The GLM models revealed differences in the type of climate variables, which influenced the abundance of certain fly species. The improvement of all models by the inclusion of the all day (24 h) minimum temperature three weeks before trapping suggests that pre-adult climatic conditions are an important determinant for abundance of adult flies. The all day (24 h) minimum temperature during the week of trapping was also an important variable for fly numbers for the Sarcophagidae species and three of the five Calliphoridae species. *O. varia* numbers are influenced mainly by temperature

with *O. varia* being the only fly species, which has its abundance affected significantly by the all day (24 h) temperature range. This is consistent with the observation that 70% of the trap catch variation in *Musca vetustissima* Walker (an Australian bushfly with similar behaviour to *O. varia* (Barratt *et al.*, 2001)) was accounted for by within day deviance of temperature (Vogt, 1986).

The GLM models explained a total of 77% of the variation in observed *O. varia* log numbers on pasture compared to only 51% under gorse. The all-day temperature range only had an effect on open pasture, which probably reflects the larger fluctuations in temperature that would be experienced in the open compared to the sheltered environment present under gorse bushes. The 20% discrepancy between the two environments for *O. varia* may be due to unmeasured gorse associated factors. Nearly twice as many individuals of *O. varia* were trapped on open pasture compared to under gorse bushes. As larvae of ovoviviparous fly species, such as *O. varia*, are known to reach full size ahead of oviparous species (Levot *et al.*, 1979), this would account for the earlier peak in *O. varia* abundance.

All other fly species were present in higher numbers under gorse bushes or on gorse edges and this probably reflects their slightly different behaviour. Calliphoridae are attracted by concentrated odours (Heath & Appleton, 2000) and feed from several sources (Oldroyd, 1964). They are fond of sweet and fermenting liquids (Zumpt, 1965) and nectar from flowering vegetation (Barratt *et al.*, 2001). However, females still need a protein-containing meal, such as a carcass, for maturing eggs (Zumpt, 1965). Successful colonisation of carcasses depends on the position of a fly in space relative to the place where a carcass is found (Kneidel, 1984), and carcass size and carcass position also influence productivity (Heath & Appleton, 2000). Depending upon where the carcasses are located, this may obscure the true fly abundance for the different vegetation types. A slower dehydration of carrion under gorse may also be of importance for larval development (Heath & Appleton, 2000).

The three species from the genera *Calliphora*, which were also abundant in winter months, had very similar climate variable patterns. The minimum all-day (24 h) temperature and the habitat were important determinants of fly abundance for two and three *Calliphora* species respectively. Wind speed resulted in a decrease of fly numbers

with very high coefficients for *C. hilli* and *C. vicina* while the night time (6 pm-6 am) humidity range three weeks prior to trapping also influenced *C. stygia* and *C. vicina* abundance, although its effect was relatively small. The model of *C. vicina* was able to predict only 19% of the observed variation and other localised factors not measured in this study (e.g. soil moisture and amount of dung produced by farm animals) may improve the prediction in these models.

The numbers of *C. rufifacies* were strongly dependent on wind speed and on the all-day (24 h) temperature minimum three weeks prior to trapping. The night-time (6 pm-6 am) humidity range in the week of trapping and three weeks prior to trapping were less important. The much higher abundance peak of *C. rufifacies* in March 2001 compared to the peak in the previous year was probably due to optimal conditions of high temperature and humidity combined with low wind speed. In common with *O. varia*, *L. sericata* abundance was strongly associated with the minimum temperature and somewhat less so by the night time humidity range. Barratt *et al.* (2001) found these two species to be the most abundant species trapped in the rabbit prone areas of the South Island of New Zealand. The mainly temperature dependent abundance of these species shown in our study is consistent with the high sightings observed by Barratt *et al.* (2001) under the dry, warm summer conditions of the Central Otago region.

Weekly average rainfall and fly catches showed high variation and no obvious relationship. Although rainfall did not appear to be a significant factor in any of the fly abundance models, it may have been partly accounted for by the humidity variable. Wind speed and rainfall were not measured directly on the study site, but 30 km away in Levin. Therefore, different values for pasture and gorse bushes could not be used in the modelling procedure. Regressors also were examined graphically for patterns of non-linear relationships, and squared transformed terms for the temperature and humidity variables were included in the models. However, no improvement of the models was achieved using these terms, despite improvements having been reported by other researchers (Vogt, 1986). Minimum and range values of the climate variables used in this study may not follow the same exponential pattern as mean temperatures used in previous works (Vogt, 1986). By using minimum and range values for temperature, the more extreme weather conditions, which would have a large impact on fly survival rates, are taken into account. Although recent studies with climate variables note the

inherent multicollinearity between climate variables (Baylis *et al.*, 1999) that can influence the accuracy of the data analysis, strong correlations between predictor variables are often neglected and analysis is conducted regardless. The factor analysis and correlation matrices used here for climate variable selection allowed the potential impact of multicollinearity to be reduced. The incorporation of time lags of the same variables also will increase the likelihood of autocorrelation. However, based on the assessment of the variance inflation factors, no evidence of this was found.

All models were re-analysed using GLM models with the variable trap included as a random effect. The random effects were not significant for different fly species and this indicated that the trap effects were not correlated with the regressors and a particular trap did not influence the number of flies caught.

The survival of RHDV is limited on open pasture, but it is extended for at least three months in animal tissue (Henning *et al.*, 2003b). Blowflies and flesh flies rely on protein sources such as decaying carcasses for their development (Zumpt, 1965) and can carry RHDV for up to eleven days after feeding on RHDV infected rabbit liver (Asgari *et al.*, 1998). Survival of RHDV in rabbit carcasses could therefore provide a persistent reservoir of virus, which could initiate new infections or even epidemics, when transmitted by flies to susceptible rabbits. Relative fly abundance may influence virus transmission, and seasonal peaks in fly abundance could correspond to the occurrence of RHD outbreaks. A pronounced RHD outbreak in the same study area occurred in February 2001 (Henning *et al.*, 2003a), when fly abundance started to rise exponentially (Figure 21). In contrast, in 2000 there was no distinct epidemic (Henning *et al.*, 2003a) and observed fly numbers were lower compared to 2001 (Figure 21). Likewise, the escape of RHDV from Wardang Island to the mainland of Australia was associated with the first warm spring days and high abundance of bush and blowflies (McColl *et al.*, 2002; Cooke & Fenner, 2002).

Although certain fly species have been linked to outbreaks (Cooke, 1999), RHDV activity is unlikely to be dependent on a specific vector. In a study in Central Otago, New Zealand, RHDV was detected on *C. vicina*, *C. stygia*, *C. quadrimaculata* and *L. sericata*, with the latter most frequently testing positive for RHDV (Heath *et al.*, 1999). Barratt *et al.* (1998) conducted a field experiment in New Zealand investigating

whether healthy rabbits exposed to insects in an area in which RHD was present could be infected with the disease. RHDV was detected by reverse transcriptase polymerase chain reaction in *Hybopygia varia* Walker (known as *Oxysarcodexia varia*) and infection in susceptible rabbits occurred. Our study revealed that during the February 2001 RHD outbreak, *O. varia* had the highest abundance, and that abundance of *L. sericata* and *C. stygia* had increased ten-fold from the previous month (Figure 21). These abundance peaks of particular species may have supported or triggered RHDV transmission and contributed to the 2001 RHD epidemic. A PCR-method for detection of RHDV in different fly species is currently being developed.

Climatic conditions could directly affect the necrophilous insect succession on carcasses, as shown in temporal patterns of rabbit carcass colonisation in northern France (Bourel *et al.*, 1999). In this study, fly species attracted to carcasses after 40 days of exposure during the spring of 1996, appeared after only 10 days exposure during the spring of 1997. The temperatures in 1997 were stable compared with the fluctuations observed in 1996. This corresponds with our observation that fly abundance was greater in late summer/early autumn 2001, when the daily temperature range was smaller and the minimum daily temperature higher, compared with the same period in 2000. When temperatures are low insects on carcasses develop more slowly thereby delaying the appearance of adult flies (Bourel *et al.*, 1999). In addition, favourable high temperatures increase the rate of carcass decomposition (Bourel *et al.*, 1999) resulting in suitable conditions for fly breeding. The presence of scavengers may also support fly breeding through the opening up of rabbit carcasses, and exposing them more thoroughly to climatic conditions (Henning *et al.*, 2003c; Henning *et al.*, 2003d).

Replication of RHDV in arthropods is unlikely (Westbury, 1996) and RHDV transmission by insects is considered to be mechanical (Cooke & Fenner, 2002). In the blowfly life cycle, eggs are usually laid around the eyes, nostrils and ears of a cadaver, followed by development of the larval form, a pupal stage away from the cadaver and adult fly emergence (Smeeton *et al.*, 1984). The conjunctivae are the preferred location for flies on live animals, and Gehrmann and Kretzschmar (1991) found that exposure to 100 RHD virus particles resulted in conjunctival infection and death of rabbits. In addition, Asgari *et al.* (1998) showed that ingestion of RHDV infected fly faeces leads to infection and death.

Dynamics of fly populations are complex as access to cadavers and prevalence of a particular insect species will influence patterns of oviposition and larviposition. The development of insects on carrion is location-specific, depending on the species present and the local climate. The only study in this context performed in New Zealand was conducted on human corpses found in the Auckland region (Smeeton *et al.*, 1984). It showed that *C. vicina*, *C. stygia* and *L. sericata* ovoposit within a few hours of death and continue to do so for at least another two weeks. In comparison, the flesh fly *Sarcophaga crassipalpis* Macquart does not appear to larviposit on bodies less than a week old. This indicates that different fly species may contribute to several stages of RHDV transmission. Furthermore decomposition and arthropod succession is probably faster in rabbit carcasses compared to human corpses because of their smaller size (Bourel *et al.*, 1999).

The role of insect transmission in the epidemiology of RHDV is uncertain, but is likely to vary due to geographic and climatic factors. In this longitudinal study, we described fluctuations in abundance of several potential vectors of RHDV in relation to observed natural epidemics of the disease in a wild rabbit population. Our findings are consistent with the hypothesis that increases in abundance of some fly species, and in particular *O. varia*, may contribute to RHDV transmission in this region, and that climatic factors affect the risk of transmission from year to year.

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Appendix 1: Climate variables created from data collected at Himatangi study site (1-24) and obtained from regional weather stations (25-28)

1. Daily average temperature (24h)
2. Daily average humidity (24h)
3. Daily maximum temperature (24h)
4. Daily maximum humidity (24h)
5. Daily minimum temperature (24h)
6. Daily minimum humidity (24h)
7. Daily temperature range (24h)
8. Daily humidity range (24h)
9. Day time (6am-6pm) average temperature
10. Day time (6am-6pm) average humidity
11. Day time (6am-6pm) maximum temperature
12. Day time (6am-6pm) maximum humidity
13. Day time (6am-6pm) minimum temperature
14. Day time (6am-6pm) minimum humidity
15. Day time (6am-6pm) temperature range
16. Day time (6am-6pm) humidity range
17. Night time (6pm-6am) average temperature
18. Night time (6pm-6am) average humidity
19. Night time (6pm-6am) maximum temperature
20. Night time (6pm-6am) maximum humidity
21. Night time (6pm-6am) minimum temperature
22. Night time (6pm-6am) minimum humidity
23. Night time (6pm-6am) temperature range
24. Night time (6pm-6am) humidity range
25. Foxton daily sunshine duration in hours
26. Levin average rainfall per day
27. Levin average wind speed in m/s per day
28. Levin gust speed in m/s per day

Appendix 2: Life cycle data obtained from the literature for two different fly species (Kamal, 1958).

Species	Mean (range) of total immature days	Mean (range) of adult life span in days
<i>Calliphora vicina</i>	18 (14-25)	25 (24-35)
<i>Lucilia sericata</i>	12 (14-25)	46 (40-59)



Unit for exposure of samples to environmental conditions



Housing of rabbits for experimental studies

Chapter 6

Survival of *Rabbit haemorrhagic disease virus* (RHDV) in the environment*

*Submitted by J. Henning, J. Meers, P. R. Davies and R. S. Morris to *Epidemiology and Infection*

SUMMARY

A study was conducted to investigate the persistence of *Rabbit haemorrhagic disease virus* (RHDV) in the environment. Virus was impregnated onto two carrier materials (cotton tape and bovine liver) and exposed to environmental conditions on pasture during autumn in New Zealand. Samples were collected after 1, 10, 44 and 91 days and the viability of the virus was determined by oral inoculation of susceptible 11 to 14-week-old New Zealand White rabbits. Evidence of RHDV infection was based on clinical and pathological signs and/or seroconversion to RHDV. Virus impregnated on cotton cloth was viable at 10 days of exposure but not at 44 days, while in bovine liver it was still viable at 91 days. The results of this study suggest that RHDV in animal tissues such as rabbit carcasses can survive for at least 3 months in the field, while virus exposed directly to environmental conditions, such as dried excreted virus, is viable for a period of less than one month. Survival of RHDV in the tissues of dead animals could therefore provide a persistent reservoir of virus, which could initiate new outbreaks of disease after extended delays.

INTRODUCTION

Rabbit haemorrhagic disease virus (RHDV) emerged in China in 1984 and spread throughout Europe in the 1980s. The virus causes a severe, systemic disease in European rabbits (*Oryctolagus cuniculus*), which is characterised by hepatocellular necrosis and disseminated intravascular coagulation. Morbidity of 100% and mortality of 90 to 95% are observed in rabbits older than 3 months of age [1-3]. In addition to a natural resistance of young rabbits (up to four weeks of age) to this disease [4-6], maternal antibodies provide protection to an age of approximately 8 weeks [7].

In most countries, research into RHDV has focused on developing methods to minimise the effects of the virus on wild, farmed and pet rabbits. However, in Australia and New Zealand, where the introduced European rabbit is a major vertebrate pest species, research has focused on finding methods to maximise the effect of the virus on wild rabbit populations so that the virus can be used as a biological control agent.

The limited knowledge on the survival of RHDV in the natural environment has been inferred from epidemiological evidence based on continuous infection and mortality

rates within wild rabbits populations [8], and the detection of virus in rabbits, flies and fly spots [9]. In laboratory-based studies, Smid and colleagues [10] investigated the survival of RHDV at various temperatures and McColl and colleagues [11] reported that RHDV remains infective in rabbit carcasses up to 30 days post death. However, there have been no published studies of the biological stability of RHDV under natural environmental conditions.

The study reported in this paper, the first in a series of two, is about the influence of environmental conditions on the survival of RHDV and on the response in rabbits to virus exposed to the environment. The aim was to gain a better understanding of the field epidemiology of this virus in its natural host by determining the duration of RHDV infectivity following exposure to typical rural environmental conditions in New Zealand. Two different carrier materials were impregnated with the virus and held in a natural environment for up to 91 days. Daily temperature and humidity values were recorded over this time period and the infectivity of the samples was measured at various time intervals by inoculation into susceptible rabbits.

MATERIALS AND METHODS

Virus

The commercial product 'RCD-ZEN' (Zenith Technology Corporation Limited, Dunedin, New Zealand) generated from RCD CAPM V-351 (Czechoslovakian strain) Master Seed Virus was used. The batch purchased for this study (Z25) had a rabbit LD₅₀ titre between 10^6 and 10^7 per ml (Max Shepherd, Zenith Technology Corporation Limited, pers. comm.).

Preparation of viral suspension and its exposure to the environment

Two vehicles were used to study RHDV survival: cotton tape and bovine liver. The cotton sample was prepared by absorbing viral suspension (0.5 ml per rabbit) onto a 12 cm piece of washed, sterilised 100% cotton tape (Trendy Trims, New Zealand) and leaving to dry. The liver sample was prepared by injecting viral suspension (0.5 ml per rabbit) into a 20 g piece of bovine liver. Untreated cotton and bovine liver samples were used as negative controls.

A special unit was designed to allow exposure of samples to environmental conditions while protecting them from damage caused by insects and animals. The units consisted of a wooden-framed box with walls of insect-proof netting (Fibreglass Flyscreen Mesh) and a pitched roof made of clear corrugated plastic sheets with a low ultraviolet absorption rating (Sunlight Light Blue, Suntuf Inc.). The roof protected samples from rainwater but allowed passage of ultraviolet light. Within the sampling unit, samples of virus-impregnated cotton tape and bovine liver were placed in open 23 x 13 cm plastic racks and the racks placed in metal cages suspended on metal chains 15 cm above the ground. Control samples were placed in an identical sampling unit and both units placed in an open pastoral environment.

To recover virus after exposure to the environment, each cotton tape and liver sample (diced) was placed in a 200 ml container, covered with resuspension medium (1 part distilled water to 4 parts serum-free Eagle's medium, 2.5 ml per rabbit to be inoculated) and left to stand at 4°C for one hour [12]. The samples were then centrifuged at 1800 g for 15 minutes and supernatants collected and filtered through 0.45 µm filters (Life Technology) followed by filtration through 0.2 µm filters to remove contaminating bacteria and fungi.

Rabbits

Eleven to fourteen week-old New Zealand White rabbits, which had not been vaccinated against RHDV, were obtained from a single commercial laboratory animal colony. Virus-inoculated rabbits were kept in a climate controlled room (17°C) and negative control rabbits were housed in a separate building on the same site. One uninoculated sentinel rabbit per trial was housed in the same room as the virus-inoculated rabbits to test for horizontal transmission of the virus.

Each rabbit was individually housed in a standard rabbit cage (56 x 44 x 45 cm) and fed *ad libitum* with commercial pelleted rabbit feed. Rabbits were acclimatised for 5 to 7 days prior to inoculation. Following inoculation, rabbits were observed continuously for 7 days, then at 4-hour intervals until 10 days post-inoculation (p.i.) and then at daily intervals until 30 days p.i.. Body weight was recorded prior to inoculation, at 5, 10, 20, and 30 days p.i. and immediately post-death.

As soon as severe signs of RHD were observed, rabbits were anaesthetised with an intramuscular injection of ketamine hydrochloride (100 mg/ml; Phoenix Pharm Distributors Limited, Auckland, New Zealand) and xylazine (20 mg/ml; Phoenix Pharm Distributors Limited, Auckland, New Zealand), and then euthanased by intracardiac injection of sodium pentobarbitone (Pentobarb 300, 300 mg/ml; National Veterinary Supplies Limited, Auckland, New Zealand). Rabbits that survived 30 days p.i. were anaesthetised and euthanased as above. Necropsies were performed on all rabbits and gross pathological observations recorded.

A rabbit was classified as being infected with RHDV (and thus to have been inoculated with infectious virus) if it:

- showed clinical signs typical of RHD (apathy, dullness, ocular haemorrhaging and cyanoses of mucous membranes, ears and eyelids, anorexia, increased respiratory rate, convulsions, ataxia, posterior paralyses) or
- showed pathological changes typical of RHD (pale yellow or greyish liver with marked lobular pattern, petechial and echymotic, multifocal haemorrhages of the lung, lung oedema, lung congestion, splenomegaly, poor blood coagulation, swollen, dull, pale to patchy reddish discolouration of the kidney) or
- tested positive for RHDV antibodies with a 1:40 dilution of serum.

We concluded that if none of these criteria were met the rabbit was uninfected and the virus in the inoculum inactivate.

RHDV antibody testing

Blood samples were collected from ear veins 3 to 5 days prior to inoculation of the rabbit, at 5, 10, 20 and 30 days p.i., and also from euthanased and dying rabbits. Blood samples were centrifuged at 1800 g for 15 minutes and the sera removed and stored at -80°C until testing. Antibodies to RHDV were assayed by AgResearch (Wallaceville Animal Research Centre, Upper Hutt, New Zealand) using a competition ELISA [13]. Samples were assayed in four-fold serial dilutions from 1:10 to 1:640. Samples were classified as RHDV positive if inhibition was $\geq 50\%$ in serum diluted 1:40.

Study design

The study was comprised of two short-term pilot experiments to develop the methodology followed by a long-term exposure trial. The objectives of the pilot experiments were to define the viral dosages and the route of inoculation to be used, to determine the suitability of the carrier materials, and to minimise the number of rabbits required in the subsequent long-term trial.

Pilot experiment 1

Experiment 1 was conducted under laboratory conditions. Virus-impregnated cotton tape and bovine liver, were prepared as described earlier and stored at 4°C for 24 hours. Virus was recovered from the carrier materials and two dilutions (10^{-2} and 10^{-3}) were prepared in serum-free Eagle's medium. For each of the three treatments (undiluted, 10^{-2} and 10^{-3}) two rabbits were inoculated by intramuscular inoculation. Negative control suspensions prepared from both carrier materials were inoculated into two rabbits each (Table 17). Rabbits were monitored for up to 30 days p.i. and assessed for RHDV infection as above.

Pilot experiment 2

Virus was impregnated on each of the carrier materials, as described above, and placed for up to 5 days in a sampling unit located in a rural environment near Dannevirke (longitude 176.095, latitude -40.214) in the North Island of New Zealand. Control samples on cotton tape and bovine liver were placed in a second unit located 10 metres away. Viral suspensions were prepared from cotton tape samples removed after 1 and 5 days and bovine liver samples removed after 5 days. Rabbits were inoculated orally by syringe with 1 ml of either an undiluted or a 10^{-2} dilution of the viral suspension or with a control preparation. Table 18 shows the number of rabbits inoculated with each suspension.

In addition, the intramuscular and oral inoculation routes were compared by inoculating 3 and 2 rabbits respectively with 0.5 ml of virus suspension that had been held at 4°C for 24 hours on cotton tape.

Long-term exposure study

The virus-impregnated cotton tape and bovine liver were placed in a sampling unit located in an open pastoral environment near Himatangi (longitude 175.317, latitude -40.400,) in the North Island of New Zealand. Control samples of bovine liver and cotton tape were kept in a unit placed in the same environment 10 metres from the sample unit. Samples were removed at 1, 10, 44 and 91 days after placement. Rabbits were inoculated orally with 1 ml of either undiluted or a 10^{-2} dilution of viral suspension prepared from the samples or with control preparation. Table 19 shows the sampling intervals and the number of rabbits inoculated with each dilution. If virus inoculated rabbits were clinically unaffected after exposure to a given treatment, the subsequent exposure interval was still evaluated for that treatment. Failure to observe clinical disease in two consecutive exposures was considered confirmation of virus inactivation for a given treatment. For each sampling interval an un-inoculated sentinel control rabbit was caged in the same room with the virus inoculated control rabbits.

Weather data recording

Gemini data loggers were placed in the sampling units described above and the temperature and relative humidity recorded at 2 minute intervals. Gemini Logger Manager Version 2.10 was used to download the data. These climate data were summarised to hourly averages and the temperature humidity indexes [14], averages, maximums and ranges for temperature and humidity were calculated for the different intervals of viral exposure.

Data analysis

Counts of surviving and dying rabbits in different treatment groups were compared using the exact estimation method in SPSS version 9.0 (SPSS Inc., Chicago, IL, USA). When multiple pairwise comparisons were conducted, the Bonferroni correction was applied [15]. The time interval to death was investigated with Kaplan-Meier Survival Plots and compared statistically between groups using log rank tests in S-PLUS for Windows version 2000 (Insightful Corp., Seattle, Washington, USA).

RESULTS

All rabbits used in the three experiments tested seronegative prior to the commencement of each study.

Pilot Experiment 1

All rabbits inoculated with 10^0 or 10^{-2} virus preparations from either of the carrier materials showed signs of viral infection or seroconverted (Table 17). Four of the eight rabbits which received these preparations died with typical RHD signs and the remaining four rabbits tested positive for RHDV antibodies on at least one sampling occasion, with titres ranging from 1:160 to $\geq 1:640$. None of the rabbits inoculated with 10^{-3} virus preparation or with the control preparation from either carrier material showed signs of RHD or seroconverted. No clinical signs or seroconversion were observed in the un-inoculated sentinel rabbit kept with the inoculated rabbits in the same room.

Table 17: Number of rabbits showing disease or sero-conversion to RHDV following intramuscular injection with virus preparations from inoculated cotton tape and bovine liver held at 4°C for 24 hours

Carrier material	Dilution	Number of rabbits infected ^a
		Number of rabbits inoculated
Cotton	10^0	2/2 ^b
	10^{-2}	2/2 ^b
	10^{-3}	0/2
Liver	10^0	2/2 ^b
	10^{-2}	2/2 ^b
	10^{-3}	0/2
Control	-	0/4

a Infection defined as presence of clinical signs and/or pathology typical of RHD and/or seroconversion (RHD antibody titre $\geq 1:40$ on one or more sampling occasions).

b One rabbit had RHD and one rabbit seroconverted without signs of RHD.

Pilot Experiment 2

All rabbits that were inoculated orally with undiluted or 10^{-2} preparations from the virus-impregnated cotton tape that had been held in the environment for 1 or 5 days, died with typical signs of RHD (Table 18). Six of the 7 rabbits inoculated with preparations from the virus-impregnated liver that had been held in the environment for 5 days, died from RHD. The remaining rabbit (inoculated with 10^{-2} dilution) did not show signs of RHD but had an antibody titre of $\geq 1:640$ at 30 days p.i.. The rabbits inoculated with control preparations derived from either carrier material, and the in-room sentinel rabbit, did not show signs of RHD and were not RHDV antibody positive at any time. All rabbits inoculated intramuscularly (3) or orally (2) with undiluted virus preparation, which had been held at 4°C for 24 hours, showed clinical signs of RHD and died or were euthanased.

Table 18: Number of rabbits showing disease or sero-conversion to RHDV following oral dosing with virus preparations from inoculated cotton tape and bovine liver held in the environment for 1 or 5 days

Carrier material	Duration of exposure	Dilution	Number of rabbits infected ^a
			Number of rabbits inoculated
Cotton	24 hours	10^0	4/4
		10^{-2}	3/3
		Control	0/2
	5 days	10^0	4/4
		10^{-2}	3/3
		Control	0/2
Liver	5 days	10^0	4/4 ^b
		10^{-2}	3/3 ^c
		Control	0/2

a Infection defined as presence of clinical signs and/or pathology typical of RHD and/or seroconversion (RHD antibody titre $\geq 1:40$ on one or more sampling occasions).

b One rabbit was seropositive at time of death (5 days p.i.).

c Two rabbits had RHD and one rabbit seroconverted without signs of RHD.

Long-term exposure study

Cotton tape: All rabbits that were inoculated orally with undiluted or 10^{-2} preparations from the virus-impregnated cotton tape that had been held in the environment for 1 day, developed RHD (Table 19). For preparations made after 10 days of environmental exposure, only the rabbits, which received the undiluted preparation were infected, and none of those that received the 10^{-2} preparation showed signs of RHD or seroconverted. For preparations made after 44 and 91 days of environmental exposure, none of the rabbits, which received either undiluted or diluted preparations showed signs of RHD or seroconverted.

Bovine liver: For the preparations eluted from bovine liver, infectious virus was still present in samples that had been exposed to the environment for 91 days. All 4 rabbits inoculated with the undiluted preparation at that sampling time developed RHD (Table 19). However, none of the 4 rabbits inoculated with the diluted (10^{-2}) preparation from 91 days exposure were infected. At 44 days exposure, 3 of the 4 rabbits inoculated with undiluted preparations developed RHD, but only 1 of the 4 rabbits, which received the diluted preparation, became infected. Rabbits inoculated with control preparations derived from either carrier material did not produce any signs of RHD or other diseases, and were not RHDV antibody positive at any sampling occasion. None of the in-room sentinel rabbits developed RHD symptoms or were sero-positive at any time.

For the cotton samples there was a significant association between duration of environmental exposure and risk of death in both the diluted ($P = 0.006$) and the undiluted groups ($P < 0.001$). Multiple pairwise comparisons for risk of death between different exposure times of cotton samples were not significant. The risk of death was higher ($P = 0.029$) for rabbits inoculated with undiluted versus diluted samples eluted from cotton tape after exposure to the environment for 10 days. There was a significant association between duration of environmental exposure of liver samples and risk of death among rabbits inoculated with diluted (10^{-2}) preparations ($P = 0.055$). Because RDHV from liver samples in the high dilution group was still infective after 91 days of environmental exposure, it was not possible to estimate the duration of infectivity in this group. The failure to detect differences between dilutions within the liver treatment groups at the $P = 0.1$ threshold for statistical significance for the shorter exposure periods is attributed to the small sample sizes.

Table 19: Number of rabbits showing disease or sero-conversion to RHDV following oral dosing with virus preparations from inoculated cotton tape and bovine liver held in the environment for 1, 10, 44 and 91 days

Carrier material	Duration of exposure	Dilution	Number of rabbits infected ^a
			Number of rabbits inoculated
Cotton	1 day	10 ⁰	4/4 ^b
		10 ⁻²	4/4
		Control	0/2
	10 days	10 ⁰	4/4
		10 ⁻²	0/4
		Control	0/2
	44 days	10 ⁰	0/4
		10 ⁻²	0/4
		Control	0/2
	91 days	10 ⁰	0/4
		Control	0/2
Liver	10 days	10 ⁰	4/4
		10 ⁻²	3/4 ^c
		Control	0/2
	44 days	10 ⁰	3/4
		10 ⁻²	1/4 ^d
		Control	0/2
	91 days	10 ⁰	4/4
		10 ⁻²	0/4
		Control	0/2

a Infection defined as presence of clinical signs and/or pathology typical of RHD and/or seroconversion (RHD antibody titre $\geq 1:40$ on one or more sampling occasions).

b One rabbit seropositive (titre $\geq 1:640$) at time of death (4 days p.i.).

c One rabbit seropositive (titre $\geq 1:640$) at 5 days p.i..

d This rabbit seroconverted (titre $\geq 1:640$) without signs of RHD at 10 days p.i..

Serology

Out of 87 virus-inoculated rabbits used in the three studies, only 9 tested positive for antibodies to RHDV on one or more sampling occasions (Table 20). Only three of 40 animals sampled at the time of death or shortly before death had antibodies against RHDV at that time. The six other sero-converting rabbits survived RHDV infection and of these rabbits, three were seropositive at 5 days p.i.. The antibody titres of two of these rabbits increased between 5 and 20 days, with the third rabbit becoming antibody negative at 10 days p.i. and remaining negative at 20 and 30 days p.i.. The other three surviving rabbits sero-converted at 10 or 20 days p.i.. The titres and sampling times are shown in Table 20.

Table 20: Antibody titres and time of death (in hours) of RHDV- inoculated rabbits

Study	Material	Exposure	Virus dilution	Time to death	RHD death	Highest level of RHDV antibodies p.i.			
						5 d	10 d	20 d	30 d
PS1	cotton	4°C, 1 d	10 ⁻²	n/a		≥1:640	neg	neg	neg
PS1	cotton	4°C, 1 d	10 ⁰	n/a		neg	neg	≥1:640	≥1:640
FS	liver	env., 44 d	10 ⁻²	n/a		neg	≥1:640	≥1:640	≥1:640
PS1	liver	4°C, 1 d	10 ⁰	n/a		neg	1:40	≥1:640	≥1:640
PS1	liver	4°C, 1 d	10 ⁻²	n/a		1:40	1:40	≥1:640	≥1:160
PS2	liver	env., 5 d	10 ⁻²	n/a		1:40	≥1:640	≥1:640	≥1:640
FS	cotton	env., 1 d	10 ⁰	98 h	≥1:640				
PS2	liver	env., 5 d	10 ⁰	126 h	n/s	1:40			
FS	liver	env., 10 d	10 ⁻²	165 h	n/s	≥1:640			
PS	pilot study				FS	final study	neg	negative	
env.	environmental placement				d	day	n/s	not sampled	
n/a	not applicable								

Survival analysis

Continuous monitoring of rabbits in the post inoculation period allowed the exact hour of death to be recorded. The survival times of inoculated rabbits are compared in Figures 23-26.

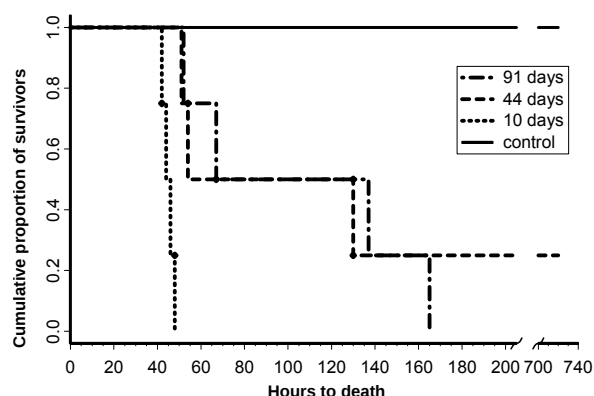


Figure 23: Kaplan Meier survival plot for hours to death of rabbits inoculated with a 10^0 RHDV dilution prepared from liver after exposure to environmental conditions. Log rank test comparing survivorship among virus inoculated rabbits = 14, $df = 2$, $P = 0.0009$.

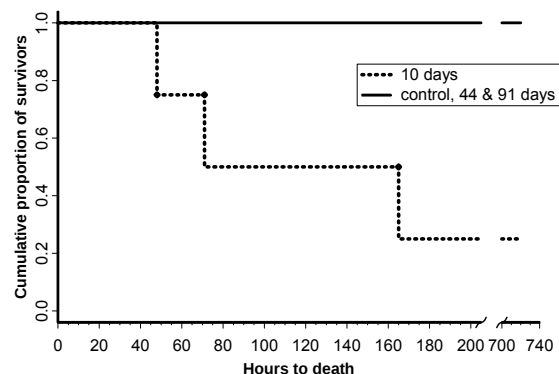


Figure 24: Kaplan Meier survival plot for hours to death of rabbits inoculated with a 10^{-2} RHDV dilution prepared from liver after exposure to environmental conditions. Log rank test comparing survivorship among virus inoculated rabbits = 8.3, $df = 2$, $P = 0.0158$.

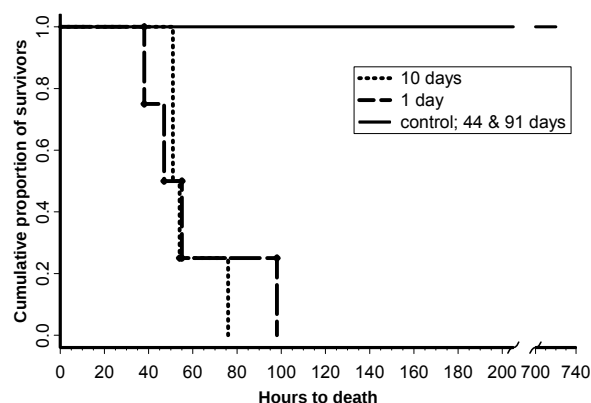


Figure 25: Kaplan Meier survival plot for hours to death of rabbits inoculated with a 10^0 RHDV dilution derived from cotton after exposure to environmental conditions. Log rank test comparing survivorship among virus inoculated rabbits = 17, $df = 3$, $P = 0.0007$.

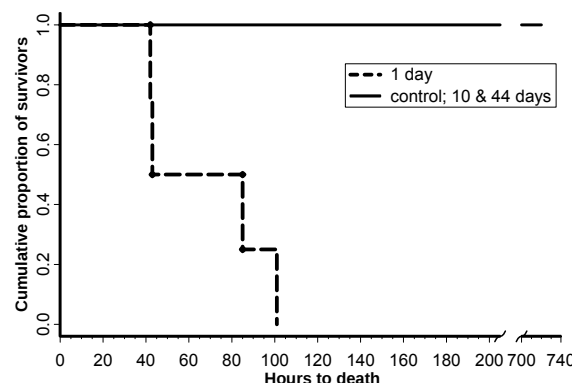


Figure 26: Kaplan Meier survival plot for hours to death of rabbits inoculated with a 10^{-2} RHDV dilution derived from cotton after exposure to environmental conditions. Log rank test comparing survivorship among virus inoculated rabbits = 14, $df = 2$, $P = 0.0009$.

Time to death was significantly prolonged in rabbits inoculated with virus that had been longer exposed to environmental conditions. As the periods of liver sample

exposure to the environment increased from 10 to 44 to 91 days, so did the time to death for the rabbits treated with the respective inocula ($P < 0.001$) (Figure 23). Inoculation of rabbits with diluted virus from liver samples kept from 10 to 44 days in the environment (Figure 24) also extended the time to death ($P < 0.016$). Cotton samples kept for longer intervals (10, 44 and 91 days for undiluted virus samples and 1, 10 and 44 days for diluted virus samples) resulted in prolonged time to death for both the undiluted (Figure 25, $P < 0.001$) and diluted (Figure 26, $P < 0.001$) inocula.

Environmental conditions

The long-term exposure study was conducted from 25 March 2001 until 24 June 2001. During this period mean ambient temperatures declined (Table 21) and the diurnal temperature range decreased by approximately 40% between days 1 and 90. Relative humidity increased during the course of the study, although the diurnal humidity range remained largely stable.

Temperature and humidity values for day-time (6 pm-6 am) and night-time (6 pm-6 am) are shown in Table 22. Temperature range was considerably broader during the day-time at the beginning of the virus exposure study, but declined to similar levels for day- and night-time by the end of the study. Relative humidity was generally higher at night, and the total range and average range of humidity was greater during the day. Humidity increased as the Southern Hemisphere winter months were approached.

Figure 27 displays the daily average and the daily range of the Temperature-Humidity Index for the period of RHDV exposure to environmental conditions. Notable are the larger variations in Temperature-Humidity Indices from day to day in periods of 10 days and 44 days of virus exposure. However, the daily average of this index was relatively stable over the whole period. Figure 28 compares the day-time and the night-time range of the Temperature-Humidity. In March and April 2001 the fluctuation of the day-time index was considerably larger than at night-time. The range of the index in May and June 2001 followed similar patterns during the day and night with lower values than in the previous months.

Table 21: Mean daily temperature (T) and humidity (H) recordings for different periods of RHDV exposure to the environment

Interval	T av (°C)	T av range (°C)	H av (%)	H av range (%)
1 d	17.6	13.4	80.4	22.5
>1 to 10 d	15.2	12.6	74.6	24.6
>10 to 44 d	12.9	13.1	85.5	22.2
>44 to 91 d	8.8	8.8	91.6	20.4
av average	d day			

Table 22: Mean day-time and night-time temperature (T) and humidity (H) recordings for different periods of RHDV exposure to the environment

Virus exposure	T av (°C)		T av range (°C)		H av (%)		H av range (%)	
	Day	Night	Day	Night	Day	Night	Day	Night
1d	19.7	15.5	13.4	6.4	78.3	82.4	22.5	5.7
>1 to 10 d	17.4	12.9	9.7	7.1	72.6	76.9	17.3	17.4
>10 to 44 d	15.2	10.7	11.3	7.7	82.3	88.7	19.6	12.1
>44 to 91 d	10.1	7.5	6.8	5.7	89.9	94.1	18.0	8.5
av average	d day							

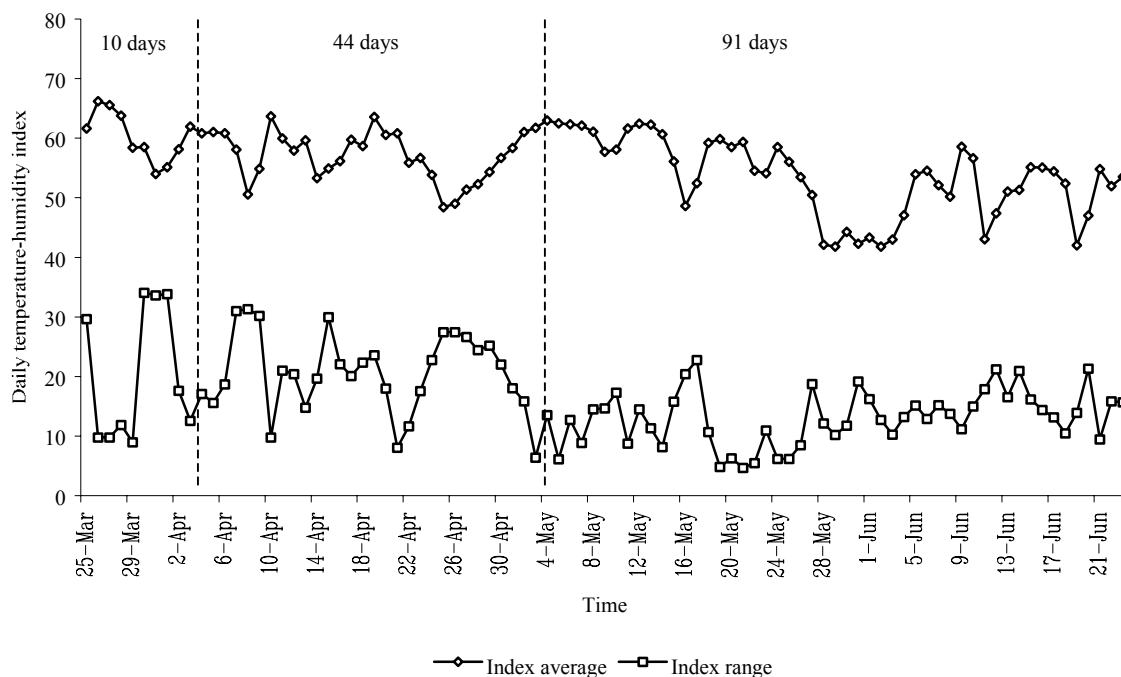


Figure 27: Daily average and daily range of the Temperature-Humidity Index for the periods of RHDV exposure to the environment

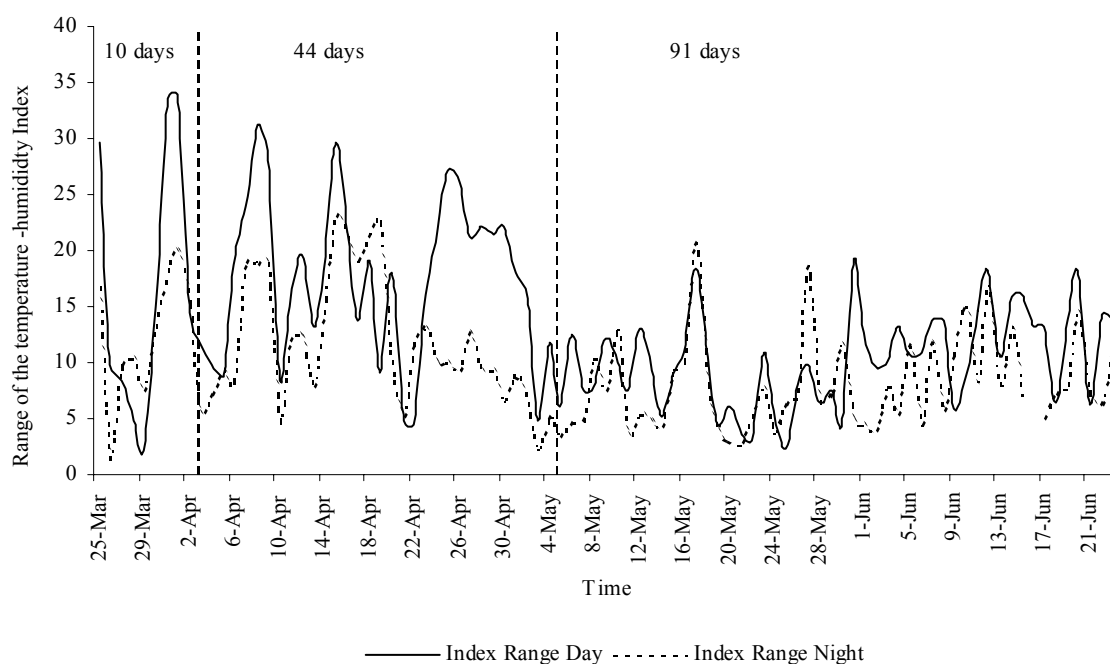


Figure 28: Day-time and night-time range of the Temperature-Humidity Index for the periods of RHDV exposure to environmental conditions

DISCUSSION

The ability of agents to survive in the environment is a key determinant of the epidemiology of infectious diseases and of the relative importance of potential routes of transmission. At the commencement of this study, there was little published information available on the survival characteristics of RHDV, although data on related caliciviruses suggested that prolonged survival under environmental conditions was likely [16-18]. The lack of methods for *in vitro* cultivation of RHDV presents a major obstacle to understanding the ability of this virus to endure under field conditions. Options for studying survival characteristics of unculturable agents include *in vitro* studies with a culturable surrogate agent that is biologically related [19], or *in vivo* studies using experimental challenge of susceptible hosts with the agent itself (as in this study). The primary concern with the use of surrogate agents is the reliability with which it is possible to extrapolate the findings across species, while the primary concern with *in vivo* studies is ethical cost. The small pilot experiments in this study were designed to verify and optimise the methods for the major study.

Our observations under natural environmental conditions indicated that virus survival was affected by duration of field exposure and the vehicle (liver or cotton) used. Virus kept dried on cotton cloth, which would mimic dried excreted virus in the field, was viable for up to 10 days of exposure to environmental conditions, but not after 44 days. Although no statistical significance could be obtained for pairwise multiple comparisons for risk of death at prolonged exposure periods for cotton samples, a clear trend of reduced mortality with extended environmental exposure was evident and considered as biologically meaningful. Failure to detect statistical significance was probably due to the small number of observations. Virus injected into bovine liver, which was used to mimic RHDV in rabbit carcasses, was still viable after 91 days. The buffer of surrounding tissue probably protected the virus from desiccation and UV light compared with the dried cotton matrix. These findings indicate that the risk of transmission of RHDV via exposure of wild rabbits to environmental reservoirs of virus is probably influenced by the nature of the source of the infecting virus. Virus in rabbit carcasses could remain infectious for at least three months, whereas virus secreted by infected rabbits into the environment may retain infectivity for less than half this time.

Ethical constraints did not permit the accurate determination of viral titres. However, to improve the ability to detect subtle loss of infectivity following environmental exposure, in addition to undiluted preparations, one RHDV dilution just able to produce infection in rabbits without environmental exposure ('borderline' dilution) was included. The pilot experiments determined the probable endpoints and confirmed that the proposed methodology was appropriate for conducting the larger field study.

The pilot experiments confirmed that both oral and intramuscular inoculation resulted in infection of rabbits. Cooke and Berman [20] also showed that the route of infection (intramuscular, intradermal and oral) with RHDV did not affect rabbit survival. The oral route was preferred for the longitudinal study, as ingestion of virus is considered to be the predominant direct transmission route for RHDV in the field [21], particularly in New Zealand where distribution of RHDV-covered baits is a common practice of rabbit control [22].

In the only detailed study published of RHDV survival, Smid and colleagues [10] showed that RHDV in tissue suspensions can survive at 60°C for 2 days, and at 4°C for 225 days. Virus from tissue suspensions dried on cloth was able to survive at 60°C for 2 days and at room temperature for 1 to 20 days, as confirmed by 100% mortality in inoculated rabbits. However, after 50 and 150 days at room temperature, only one of two rabbits inoculated with the same virus from dried cloth succumbed to the disease. These observations were obtained under laboratory conditions, and conclusions for field epidemiology of RHD are limited. McColl and colleagues [11] kept rabbit carcasses at 22°C under laboratory conditions and collected liver samples up to 30 days post death. Samples taken up to 20 days post death were able to infect and kill susceptible rabbits, while samples collected after 26 and 30 days did not result in mortality and only some rabbits seroconverted. In another study reverse transcriptase polymerase chain reaction techniques showed that bones from RHDV-seropositive rabbits exposed to environmental conditions contained detectable amounts of RHDV RNA for up to seven weeks, but the viability of virus was not tested in challenge experiments [23].

The risk of epidemics of RHDV in wild rabbit populations will be influenced by the relative proportion of susceptible individuals in the population. Field data (Henning, unpublished observations) suggest annual RHD outbreaks occur at the end of the rabbit breeding season. Persistence of viable virus in the environment between inter-epidemic

intervals is unlikely as was shown by the loss of RHDV infectivity from cotton cloth samples and by the decline in viral infectivity from liver samples over a period of 3 months in this study. RHD outbreaks are most likely when the proportion of antibody negative, susceptible rabbits reach a critical level and re-contamination with RHDV occurs.

Flying insects also have been strongly implicated as mechanical vectors of RHDV [24, 25]. Viral persistence in flies that feed on rabbit carcasses could provide an alternative mechanism for viral persistence in an ecosystem between consecutive epidemics. Surviving and sero-converting animals, which represented only 7% of virus-inoculated animals in this study, may also be a virus source for infection. However, a detailed study on viral persistence in recovered rabbits is yet to be conducted. The outcome of infection with RHDV was influenced by the virus concentration, which was approximated by using two dilutions of the inocula. Therefore interpretations of RHDV infectivity and antibody responses [26] need the virus concentrations to be taken into account. Reduction of infectivity occurred between 1 and 10 days for cotton samples, and was first detected between 10 and 44 days for the liver samples.

Continuous observation of rabbits following infection allowed precise recording of incubation and survival times. Cook and Berman [20] provided relatively imprecise estimates of survival times, because animals were observed only at 8 hour intervals following exposure, and estimated survival times were based on temperature of the cadaver and the rabbit behaviour when last seen alive. In our study, reduction in infectivity was reflected in the survival time of infected rabbits following challenge. Animals exposed to lower concentrations of virus took longer to show clinical signs and to die, suggesting that the incubation period of RHD is related to viral dose. Rabbits inoculated with samples that had been exposed to the environment for longer durations had longer incubation periods suggesting that the amount of infectious virus remaining in the sample was reduced. Similar correlations between the length of the incubation period and the virus dose have been described for *Rabies virus* [27] and the *Measles virus* vaccine [28].

RHD has a very high mortality rate of 95-100% [29]. In the absence of sudden death affected rabbits deteriorate and progressively lose body weight before eventually

succumbing to the disease. In most experimental RHDV inoculation studies, viral antigen was detected post death using virus capture ELISAs on liver tissue [20] as death occurs too suddenly to allow time for blood collection for antibody detection. Intensive monitoring enabled us to bleed animals immediately before death occurred and only 8% of rabbits (3 animals) had developed antibodies at the time of death. Gavier-Widen [30] was not able to detect any antibodies using the haemagglutination inhibition test on rabbit sera sampled immediately before death. She suggested that the time interval was too short to develop antibodies as all animals in this study died or were euthanised between 12 and 106 hours post inoculation. However, the shortest interval between inoculation and death of a seropositive rabbit in our study was 98 hours post inoculation. Plassiart and colleagues [31] noted biological signs of disseminated intravascular coagulation and early necrotic changes of liver tissue as early as 30 hours post inoculation. Therefore, it seems possible that the pathogenesis of RHD allows in some cases antibody development after extremely short intervals post inoculation. Antibody detection in survivors (and in the two other sero-converting and dying animals) was observed as early as 5 days post inoculation. This is the same time period that Shien and colleagues [32] observed after experimental infection. However, Shien's experiment was conducted with only two 4-5 week old rabbits, which would have survived anyway because of their age related resilience [1, 6].

As only infectious virus will result in an antibody response [33], viral replication in the body is necessary to obtain a constantly high antibody level. Antibody titres of the five surviving sero-converting rabbits reached their highest levels between 10 and 20 days, while Shien and colleagues [32] detected antibody peaks three weeks post inoculation. In our study, four animals retained high antibody titres until the conclusion of our study at 30 days, while one animal had declining antibody titres after 5 days and another at 20 days. It is possible that rabbits with declining antibody titres had eliminated the virus and thus had no antigenic stimulation to maintain antibody levels. Shien and colleagues [32] indicated that virus could gradually be cleared from infected rabbits, resulting in a disappearance of the viraemia, but with virus persistence in bile and the spleen. However, none of the young rabbits in Shien's experiment (4-5 weeks old at time of inoculation) showed any antibody decline during their study.

No evidence of airborne transmission of RHDV was shown in this study as none of the un-inoculated rabbits ($n = 5$) kept with virus infected animals in the same room in each trial developed RHD symptoms or antibodies against RHDV.

This study was conducted in the Southern Hemisphere's autumn. Environmental conditions change with the seasons, but quantification of the impact of climate factors is difficult as they change from year to year. Climate comparisons between years and regions are most productive over longer time periods. Exposure to the UV light and fluctuations in temperature and humidity may have the largest effect on virus survival. Our study was conducted over the autumn months in 2000 and revealed changing weather conditions from a large temperature-humidity range at the beginning of the study towards a more stable, but more humid climate. Kovaliski [34] mentioned that the survival of the virus is probably shorter in the hot summer months in Australia. Higher survival rates of rabbits are found in more cool, wet regions of Australia [35] while in the lower North Island of New Zealand RHD outbreaks are occurring in the drier period of the year (Henning, unpublished data). These observations suggest that some environmental or other conditions trigger the event of an RHD outbreak and influence mortality rates. In addition to affecting viral survival, weather conditions also influence rabbit breeding, rabbit behaviour and the abundance of flying insects which are all important mechanisms for generating RHD outbreaks.

The results of this study suggest that carcasses from rabbits dying from RHDV are likely to be the reservoir for persistence of RHDV following an outbreak. Excreted RHDV from infected animals or virus coated on baits may have reduced infectivity within one to two weeks and probably do not persist longer than several weeks.

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Chapter 7

Exposure of rabbits to ultraviolet light-inactivated *Rabbit haemorrhagic disease virus* (RHDV) and subsequent challenge with virulent virus^{*}

^{*}Submitted by J. Henning, J. Meers and P. R. Davies to *Epidemiology and Infection*

SUMMARY

This study investigated whether exposure to inactivated *Rabbit haemorrhagic disease virus* (RHDV) can produce an antigenic response in rabbits and protect them from a subsequent challenge with virulent virus. The aim was to determine if the spreading of baits containing RHDV, which is a common management practice in New Zealand to reduce rabbit numbers, could result in protective immunity in wild rabbits. RHDV was inactivated by ultraviolet light using an electronic UV crosslinker with a UV dose of 168.48 W-s/cm² and a UV intensity of 0.0078 W/cm². Two groups of four rabbits were then inoculated with inactivated virus via oral and intramuscular routes. Rabbits were monitored for 30 days post inoculation and then challenged orally with virulent virus.

No rabbit exposed to inactivated RHDV developed clinical signs of RHD or had antibodies at day 30 post infection and all animals died within 82 hours after challenge with virulent virus. No antibodies were detected at the time of death. These findings suggest that exposure to UV-inactivated virus in the field or on baits will not protect rabbits against challenge with virulent virus.

INTRODUCTION

Introduced European rabbits (*Ortycolagus cuniculus*) are a major vertebrate pest in New Zealand and Australia. The use of *Rabbit haemorrhagic disease virus* (RHDV) for biological control of rabbits is a common practice in New Zealand. In rabbit infested areas, a commercial RHDV product ('RCD-ZEN', Zenith Technology Corporation Limited, Dunedin, New Zealand) is typically distributed on baits to initiate RHDV epidemics. However, not all baits are taken up by rabbits, and residual RHDV baits may undergo prolonged exposure to environmental conditions. O'Keefe and colleagues [1] suggested that large-scale use of baits coated with RHDV, which might be inactivated in the environment, could induce protective immunity of rabbits and reduce the effectiveness of biological control programs.

Environmental factors such as changing temperatures, humidity and sunshine are likely to influence the survival and infectivity of RHDV on baits. In particular, exposure to ultraviolet radiation from sunlight is likely to be an important determinant of the duration of virus infectivity. In a field study of RHDV survival, dried virus exposed

directly to sunlight on cotton cloth remained infective for susceptible rabbits for greater than 10 days, but less than 44 days [2]. In contrast, under identical environmental conditions, RHDV injected into organic tissue (liver) and therefore not exposed to direct sunlight, remained infective for at least three months [2]. The possibility that environmentally degraded RHDV virus could induce protective immunity in wild rabbit populations has considerable implications for the efficacy of biological control using this agent. This is the second of two studies investigating the influence of the environment on RHDV properties. We conducted experimental investigations to determine whether exposure to UV inactivated RHDV virus would induce seroconversion of rabbits and protect against exposure to virulent virus.

MATERIALS AND METHODS

Animals

New Zealand White rabbits aged between 10 and 11 weeks old were purchased from a commercial laboratory colony and housed in standard rabbit cages (56 x 44 x 45 cm) in climate-controlled rooms at 17°C. They had not been vaccinated against RHDV and were tested immediately prior to the study to confirm the absence of RHDV antibodies. Rabbits were fed ad libitum with commercial rabbit pellets and had constant access to water.

RHDV inactivation

A commercial product 'RCD-ZEN' (Zenith Technology Corporation Limited, Dunedin, New Zealand) that was produced from RCD CAPM V-351 (Czechoslovakian strain) Master Seed Virus was used for the study. The batch purchased for this study (Z25) had a rabbit LD₅₀ titre of approximately 10⁶ per ml (Max Shepherd, Zenith Technology Corporation Limited, pers. comm.).

The RHDV product was exposed to ultraviolet light using an electronic UV crosslinker (CEX-800, Ultralum, Inc., 6421 East Alondra Blvd, Paramount, California, USA). UV crosslinkers are designed especially to provide uniform irradiation with short wave UV light (254 nm) for crosslinking DNA and RNA. A high dose of UV exposure was chosen, based on the resilient properties of RHDV [3] and published reports on the use of UV energy to inactivate viruses and sterilise or disinfect effluents. The samples were exposed to a UV dose of 168.48 W-s/cm² with a UV intensity of 0.0078 W/cm².

Experimental Design

Two groups of four rabbits were inoculated with 1 ml of undiluted UV-inactivated virus; one group was inoculated orally and the other group intramuscularly. Thirty-five days post inoculation with inactivated RHDV, rabbits were challenged orally with approximately 10^4 LD₅₀ RHDV (RCD-ZEN) in a volume of 1 ml. A positive control group of two rabbits, not previously inoculated with inactivated virus, was also challenged with virulent RHDV, and two negative control rabbits were dosed orally with saline solution. Blood samples were collected prior to commencement of the study, at 5, 10, 20 and 30 days after inoculation with inactivated virus, and at 5, 10, 20 and 30 days post challenge with virulent virus, and at the time of euthanasia. If an animal was seronegative at 30 days following inoculation, samples from 5, 10 and 20 days post inoculation were not tested. Blood samples were centrifuged for 15 min at 1800 g to separate the sera. The sera were tested for antibodies to RHDV with the Capucci-competition ELISA [4] by AgResearch (Wallaceville Animal Research Centre, Upper Hutt, New Zealand). Four-fold serial dilutions from 1:10 to 1:640 were assayed. Samples were classified as RHDV positive if inhibition was $\geq 50\%$ in serum diluted 1:40.

Assessment of outcomes

Following inoculation with the inactivated virus, rabbits were observed several times daily for clinical signs. Following challenge with virulent virus, rabbits were observed continuously by an observer for the first 7 days and then at 4-hour intervals until 10 days post challenge (p.c.), followed by once daily until 30 days p.c.. Clinically affected rabbits were anaesthetised with an intramuscular injection of ketamine hydrochloride (100 mg/ml; Phoenix Pharm Distributors Limited, Auckland, New Zealand) and xylazine (20 mg/ml; Phoenix Pharm Distributors Limited, Auckland, New Zealand) as soon as signs of RHD [2] were observed, and then euthanased by intracardiac injection of sodium pentobarbitone (Pentobarb 300, 300 mg/ml; National Veterinary Supplies Limited, Auckland, New Zealand). Necropsies were performed on all rabbits and gross pathological observations were recorded. The presence of pathological changes typical of RHD (pale yellow or greyish liver with marked lobular pattern, petechial and echymotic multifocal haemorrhages of the lung, lung oedema, lung congestion, splenomegaly, poor blood coagulation and swollen, dull pale to patchy reddish discolouration of the kidney) was interpreted as confirmation of RHD.

RESULTS

All animals were seronegative before inoculation. None of the rabbits inoculated with irradiated virus developed clinical signs of RHD or had detectable antibodies to RHDV 30 days post inoculation (Table 23). Challenge with virulent virus resulted in clinical signs and pathology typical of RHD in all rabbits, including those previously inoculated with irradiated virus. The mean (SD) time to death post challenge was 52.9 (11.2) hours. No antibodies were detected at the time of death in any rabbit. The two negative control rabbits did not show any signs of disease and were negative for RHD antibodies 30 days p.i..

Table 23: Serology and mortality results after inoculation of rabbits with inactivated RHDV followed by challenge with virulent RHDV

Group	Sex	AB at 30 days p.i.	Time to death p.c.	AB at time of death
1. Inactivated RHDV:	female	neg	53 h	neg
IM route	female	neg	82 h	neg
	male	neg	47 h	neg
	male	neg	45 h	neg
2. Inactivated RHDV:	female	neg	42 h	neg
Oral route	female	neg	55 h	neg
	male	neg	55 h	neg
	male	neg	54 h	neg
3. Positive control:	female	n/a	46 h	neg
RHDV challenge only	female	n/a	50 h	neg
4. Negative control:	female	n/a	n/a	n/a
Saline challenge only	male	n/a	n/a	n/a

Rabbits in groups 1 and 2 were inoculated with inactivated RHDV via intramuscular (IM) and oral routes respectively. Groups 1, 2 and 3 were orally challenged with virulent RHDV 35 days later.

p.i.	post inoculation with inactivated RHDV	p.c.	post challenge with virulent RHDV
AB	antibodies	neg	ELISA titre < 1:10
n/a	not applicable		

DISCUSSION

Ultraviolet light at a wavelength between 100 and 280 nm is considered to be 'germicidal'. It damages the DNA and RNA of bacteria, viruses and other pathogens and thus destroys their ability to multiply and cause disease. It does this by eliciting single photon photochemical effects in nucleic acids through forming covalent bonds between certain adjacent bases [5]. This modification results in incorrect codes being transmitted from the nucleic acids and causes irreversible damage to the microorganisms. Inoculation with UV light inactivated RHDV did not produce any antibody response in the rabbits, and they were not protected against challenge with virulent virus.

Considering these results, it is unlikely that inactivated RHDV on baits, in rabbit carcasses or excreted into the environment will produce an antibody reaction in naive rabbits. Thus, rabbits are likely to be fully susceptible to further RHDV epidemics in the field. Inactivated RHDV administered by the oral route (which would mimic ingestion of virus) or administered intramuscularly (imitating a stinging or biting insect) will not induce a protective antibody titre. O'Keefe and colleagues [1] proposed that inactivation of virus after large-scale baiting with RHDV may result in seroconversion of rabbits and protective immunity. However, the current experimental study showed no evidence to support this hypothesis. Seroconversions in rabbits following baiting operations is more likely to result from contact of rabbits to sub-lethal doses of virulent virus.

High titres of RHDV antibodies in surviving rabbits does not necessarily indicate immune protection of rabbits as suggested by O'Keefe and colleagues [1]. Exposure to RHDV in new epidemics can produce re-infection in animals that have survived previous outbreaks [6]. Persistence of RHDV antibodies over longer time periods has not been well documented and is worthy of further investigation in longitudinal studies.

RHDV antibody responses have been documented in experimental studies by feeding foxes RHDV-infected rabbit carcasses [7] and by immunisation of rabbits with recombinant virus-like particles and structural virus proteins [8, 9]. In these studies, the ability to induce antibodies (in foxes and rabbits) may be attributable to the large, multiple doses of antigen administered, the use of adjuvants, or the chemical stabilization of the antigen. None of these factors are present in a true field situation.

These shortcomings are addressed in the current study, which more closely mimics field conditions.

The death rate of microorganisms after UV light exposure decreases with increasing humidity [10]. The rate of inactivation of RHDV in the field will depend on the specific meteorological conditions, but the quantification of these influences is difficult. In addition, UV radiation has increased over the recent years in New Zealand [11], especially the DNA and plant damaging UV light. There are regional and seasonal differences in UV radiation even within New Zealand, so that the north of New Zealand receives up to 25% more UV than the south [12].

It is possible that the antigenic structure of the virus was destroyed by the inactivation dose and method used in this experiment. The UV dose or exposure is defined as the UV light intensity multiplied by the exposure time [10]. Table 24 shows some inactivation dosages with UV light of 254 nm for a range of organisms. Inactivation of virus by UV radiation was examined as a potential method for sterilisation of blood products by Prodouz and colleagues [13]. UV light at 308 nm (UVB 308) with an exposure dose of 21.5 W-s/cm² and an intensity of 170,00 W/cm² causes minimal damage to platelets, while the use of UVB308 at a higher intensity (1,400,000 W/cm²) over a similar range of exposure does not enhance viral inactivation, but resulted in increased damage to platelets and plasma proteins.

Table 24: UV energy dosages (microwatt-seconds per square centimetre) required to inactivate selected organisms [14]

Organism type	Organism	UV energy in mW-s/cm ²
Bacteria	<i>Clostridium tetani</i>	23-36
Mold spores	<i>Aspergillus niger</i> (black)	333-468
Protozoa	<i>Paramecium</i>	200-315
Virus	<i>Rotavirus</i>	24-29
	<i>Tobacco mosaic virus</i>	440- 720

UV inactivation with wavelengths of 254 nm is commonly applied in the wastewater industry. Currently UV dosages of 60-90 mW-s/cm² are necessary to inactivate certain

human pathogenic viruses, such as *Poliovirus*, *Rotavirus* and *Hepatitis A virus*, while MS-2 bacteriophage shows more resistance [15]. Ho Chu-Fei and colleagues [16] have shown that a UV exposure dose of 65 mW-s/cm² is required in a water pollution control plant to achieve the target coliform level 95% of the time. A 95% inactivation of human enteric Adenovirus type 40 occurs with UV doses of 103 mW-s/cm² in treated groundwater [17]. It has been shown also that a UV dose of 13 mW-s/cm² is able to reach 99% inactivation of Feline calicivirus (used as a surrogate to monitor the not-culturable Norwalk virus) in treated drinking water [17].

This study has shown that exposure of rabbits to inactivated RHDV will not protect them from further challenge with viable RHDV virus. The high inactivation dose and intensity chosen in this experiment is appropriate considering the resilient properties of RHDV. However, the influence of UV light on the antigenic structure can be determined only with further challenge experiments using stepwise decreased UV light exposure and intensity.

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Chapter 8

Attitudes of New Zealand farmers to methods used to control wild rabbits^{*}

^{*}Submitted by J. Henning, C. Heuer and P. R. Davies to *Preventive Veterinary Medicine*
Additional information is enclosed at the end of the chapter in Appendices 3 and 4

Abstract

Four years after the release of *Rabbit haemorrhagic disease virus* (RHDV) in New Zealand, we conducted a mail survey of farmers to ascertain their attitudes and practices with respect to rabbit control. A multistage sampling frame, stratified by rabbit-proneness and farm type, was used to select 828 farms in 8 geographical regions. Data were analysed using survey commands in STATA 7.0. The useable response rate of the survey was 69.3%, and 21% of respondents considered rabbits to be a problem on their farm. Although practices for rabbit control had changed from 1995 to 2001, shooting (practised by 85% of respondents) remained the predominant method employed, albeit less frequently than in 1995. Ten percent of farmers used RHDV baiting, and of those 90% released the virus relatively infrequently. Farmers perceived shooting to be the most humane and environmentally safest method, while RHDV was perceived to be the most effective. Perception of the level of competition for grazing between rabbits and livestock was the factor most strongly associated with the use of shooting and RHDV. A majority (60%) of respondents considered the introduction of RHDV to have been beneficial. We conclude that farmers perceive the use of RHDV for rabbit control as an effective and ethically acceptable method. Methods other than shooting were regarded as less effective or less acceptable than rabbit haemorrhagic disease (RHD).

Introduction

Rabbits were introduced to New Zealand in the mid 1800s as an alternative food source for sailors and early settlers, and for recreational hunting. By the beginning of the 20th century, rabbit numbers had increased to a level where they had become a major pest. Rabbits compete with livestock for grazing, predispose the ground to soil erosion through burrowing and reducing plant cover, and adversely affect pasture quality (Gibb and Williams, 1994). The annual costs of rabbit control in 1995 were estimated to be greater than NZ\$ 22 million (Parkes, 1995). Coordinated efforts to control rabbits were initiated in 1947 under the auspices of the Rabbit Destruction Council and continued until governmental funding declined in the mid eighties when farmers were encouraged to take increased responsibility for rabbit control on their land (Gibb and Williams, 1994). Conventional control methods include shooting, poisoning, and trapping, all of which are labour intensive and expensive.

In the 1980s, rabbit haemorrhagic disease (RHD), a novel viral disease, caused widespread mortality of farmed and wild rabbits in China and Europe (Ohlinger et al., 1993). The epidemiological characteristics of these outbreaks (rapid transmission, high morbidity and mortality rates) presaged the use of the virus for biological control of pest rabbit populations. Unintentional release of *Rabbit haemorrhagic disease virus* (RHDV) in South Australia led to a dramatic reduction in wild rabbit populations (Mutze et al., 1998). Despite an application by New Zealand farmers to introduce RHDV being denied by the government in July 1997, the disease was deliberately released in the South Island the following month. Subsequently, the intentional spread of the RHDV was legalised in New Zealand, and RHDV became commercially available for rabbit control (Sanson et al., 2000). Before the application to introduce RHDV was denied, surveys to assess public attitudes to biological control of pests revealed widespread recognition of RHD (Fitzgerald et al., 1996; Wilkinson and Fitzgerald, 1998). However, opinions of farmers, the group most affected by and responsible for the control of rabbits, have not been systematically evaluated. In 2001, four years after RHDV was introduced into New Zealand, we conducted a mail survey of 828 farmers in eight regions of New Zealand to determine trends and current practices of rabbit control, and to ascertain opinions about RHDV and other control methods. We hypothesised that both attitudes and practices related to rabbit control were likely to vary between types of farm enterprises and with the extent of the rabbit problem that farmers had to confront. These factors were major considerations in the design and analysis of the survey, which focused primarily on pastoral livestock producers.

Materials and Methods

A key objective of the study was to compare the attitudes to, and practices of, rabbit control from farmers with significant rabbit problems, with farmers who had relatively minor rabbit problems. Particular emphasis was given to assessing opinions of farmers engaged in the major pastoral livestock industries. A multistage spatial sampling frame was developed stratified according to both rabbit-proneness and type of enterprise.

Selection of regions

1. Level of rabbit-proneness (the propensity of land to sustain rabbit populations): A digital map of New Zealand identifying 5 categories of rabbit-proneness (Kerr and

Ross, 1990) was used as the primary spatial stratum. The New Zealand Mountain Lands Institute, Lincoln University, New Zealand, conducted this classification for the Ministry of Agriculture and Fisheries of New Zealand. Classification of proneness was based on information about topography, geological formations, soil erosion, vegetative features and capability of land for agricultural production. This was then validated and combined with rabbit count information. Rabbit-proneness was classified into categories 'extreme', 'high', 'medium', 'low' and 'negligible'.

2. Region: Using ArcView 3.1 (1992-1998 Environmental Systems Research Institute, Inc., Redlands, CA, USA), the digital rabbit-proneness map was overlaid on a regional map of New Zealand. To enable comparison of farmers living in areas with differing rabbit-proneness and likely experience with RHD, eight regions were selected based on the predominant agricultural activities and rabbit-proneness (Table 25).

Table 25: General characteristics of regions selected for surveying

	Region	Main agricultural activities	Rabbit-proneness	Other
North Island	Waikato	Dairy	Low	
	Hawke's Bay	Horticulture, sheep	Moderate	Anecdotal evidence that RHD has been ineffective
	Manawatu	Beef, mixed livestock	Moderate	Epidemiological research on RHD
	Wairarapa-Wellington	Beef, mixed livestock	Moderate	
South Island	Marlborough	Horticulture (viticulture)	Moderate to high	Anecdotal evidence that rabbits cause severe losses in horticulture
	Canterbury	Sheep, mixed livestock	Moderate to high	Region with second highest rabbit-proneness
	Otago	Sheep, horticulture, mixed livestock	High	Region with highest rabbit-proneness
	Southland	Beef, mixed livestock, dairy	Moderate	

Classification of farms

The New Zealand agricultural database ('Agribase') is a register of all commercial farms and their locations in New Zealand (Sanson and Pearson, 1997), with farms classified into 33 different types. For the purpose of stratified sampling within regions, we simplified the number of farm categories to be sampled using the following procedures:

- All farm types with less than 100 records in Agribase (e.g. beekeeping, dogs, ratites) were excluded ($n = 279$ farms).
- All farm types with ambiguous definitions or considered unimportant for the study (e.g. lifestyle blocks, tourism, native bush) were excluded ($n = 20,781$ farms). Predominant categories in this group were lifestyle blocks (8,591), 'unspecified' (5,372) and 'other' (4,489).
- Farm types engaged in similar or related activities were aggregated to yield a smaller number of farm categories. 'Dairy' and 'dairy dry stock' types were aggregated into a single entity; all livestock enterprises (e.g. deer, horses, pigs and poultry) not identified as dairy, sheep, beef, or mixed (sheep-and-beef), and farmers grazing other people's livestock, were aggregated into a category of 'other livestock'. Similarly, arable cropping and seed production, flowers, forestry, fruit growing, plant nurseries, vegetable growing and viticulture were aggregated into a single 'non-livestock' category.

This process excluded 21,060 farms and identified a population of 82,608 farms in New Zealand. A total of 51,716 farms among 6 categories of farm type (Table 26) (62.6% = study population) were situated in the regions selected for the survey, and 30,892 farms (37.4%) were in other regions.

Table 26: Distribution of New Zealand farms by selected farm types

Description	Farm code	All NZ farms	Farms in study regions
Beef cattle farming	BEF	20,779	12,517
Dairy cattle farming	DAR	15,558	10,261
Non-livestock ^a	NLS	15,385	5,705
Other livestock farms ^b	OLF	10,118	6,089
Sheep farming	SHP	11,635	9,874
Mixed sheep-and-beef farming	SNB	9,133	7,270
TOTAL		82,608	51,716

^a Including arable cropping and seed production, flowers, forestry, fruit growing, plant nurseries, vegetable growing and viticulture.

^b Including deer, horse farming and breeding, poultry, pig production and goat farming.

To determine the rabbit-proneness classification for each farm, geographic centroids of farms were plotted on the map of rabbit-proneness using the X-Tools extension (Department of Forestry, Oregon, USA) for ArcView 3.1. Thus all farms were categorised by both farm type and rabbit-proneness. Due to logistic constraints, the total number of farmers that could be surveyed was approximately 800 (i.e. 100 per region). Power analysis indicated that this sample size would be adequate to detect differences in proportions as small as 0.1 compared with a null proportion of 0.5 (power of 0.8 with $\alpha = 0.05$).

Selection of farms within regions

Rabbit-proneness was the primary criterion for the stratification of farms. However, areas of extreme or high proneness occurred in only two of the eight regions, and the large majority of farms were in areas of low or negligible rabbit-proneness. Similarly, farm types were not evenly distributed among regions. Therefore, fixed proportional sampling of all categories was not possible across regions. Consequently, in each region 100 questionnaires were allocated to the negligible, low and medium categories of proneness at fixed ratios of 2:4:4 respectively. A further 80 questionnaires were assigned to farms in regions of high and extreme rabbit-proneness at a 1:1 ratio so that they would be sampled at a higher intensity. Selection of farm types was also weighted to yield a higher representation of enterprises thought likely to be more affected by the

impact of rabbits. Specifically sheep, mixed sheep-and-beef, and non-livestock enterprises were sampled twice as intensively as dairy farms and 'other livestock' farms.

The weighting matrix, the numbers of surveys allocated by farm type within each rabbit-proneness stratum, and the actual numbers of questionnaires mailed (and returned) are presented in Table 27. A programme was written in Visual Basic for Applications in Access 2000 (Microsoft Corporation) to randomly select the farms according to the specified weightings from the 51,716 eligible farms in the 8 selected regions without replacement. However, target numbers for some categories of farms in the medium, high and extreme rabbit-proneness areas were not achievable and all eligible farms in these strata were surveyed (Table 27). In fact in one area there were no eligible farms within the strata; there were no dairy farms located in areas of extreme rabbit-proneness.

Table 27: Matrix showing proportional weights (farm type by rabbit-proneness) and number of questionnaires targeted, mailed and returned (the last two in parentheses).

Rabbit-proneness							
		Negligible [‡]	Low [‡]	Medium [‡]	High [‡]	Extreme [‡]	TOTAL
Farm type	<i>proportion</i>	<i>0.2</i>	<i>0.4</i>	<i>0.4</i>	<i>0.5</i>	<i>0.5</i>	
Beef cattle	<i>0.2</i>	32	64	64	8	8	176
		(32/19)	(64/46)	(56/38)	(8/4)	(3/2)	(163/109)
Dairy cattle	<i>0.1</i>	16	32	32	4	4	88
		(16/12)	(32/24)	(24/14)	(2/1)	(0/0)	(74/51)
Non-livestock	<i>0.2</i>	32	64	64	8		176
		(32/21)	(64/44)	(55/37)	(7/6)	(4/4)	(162/112)
Other livestock	<i>0.1</i>	16	32	32	4	4	88
		(16/10)	(32/25)	(32/22)	(4/2)	(2/0)	(86/59)
Sheep farming	<i>0.2</i>	32	64	64	8	8	176
		(32/25)	(64/42)	(59/50)	(8/6)	(8/7)	(171/130)
Sheep-and-beef	<i>0.2</i>	32	64	64	8	8	176
		(32/24)	(64/50)	(60/46)	(8/4)	(8/8)	(172/132)
TOTAL		160	320	320	40	40	880
		(160/111)	(320/231)	(286/207)	(37/23)	(25/21)	(828/593)

[†] Fixed ratios 4:4:2 for sampling medium, low and negligible categories across 8 regions (not achieved due to insufficient farms in medium category in some regions)

[‡] Target weights for extreme and high categories across 8 regions (not achieved due to insufficient farms in some regions)

Statistical analysis

The survey analysis commands in Stata 7.0 (Stata Statistical Software, College Station, Stata Corporation 2001) were used to account for sampling weights (weight matrix), sampling strata (rabbit-proneness) and clustering (farm types). The incorporation of sampling weights allows the calculation of unbiased estimates, while consideration of stratification and clustering produces correct standard errors (Stata Corporation, 2001). From our multistage design of data collection we derived design-based estimates with corrected point estimates and corrected standard errors.

Survey two-way tabulations were used to assess the effect of region, farm type and rabbit-proneness on the outcome variable (use of a particular rabbit control method). To account for the survey design a second order corrected F statistic with non-integer degrees of freedom was produced from the χ^2 Wald statistic, which has the same interpretation as the Pearson χ^2 statistic (Rao and Scott, 1981, 1984). Paired analysis using the marginal homogeneity test (PROC Freq, The SAS System for Windows 8.2, SAS Institute Inc., Cary, NC, USA) was employed to evaluate the change in the level of rabbit damage and in the use of rabbit control methods from 1995 to 2001 (Stuart, 1955; Maxwell, 1970).

Univariable analysis using logistic regression was used to screen the relationship between explanatory variables and the use of specific rabbit control methods. Outcome variables were binary ('never used' or 'used') for use of poisons (1080 and Pindone), trapping and RHD baiting. For shooting, the outcome variable had three levels ('never', 'seldom' or 'often used'). Explanatory variables screened were the perceived damage caused by rabbits, and the perceived humaneness, safety for the environment and efficacy of each control method. Variables associated with the outcome variable at $P < 0.05$ were used in the multivariable analysis, and logistic regression models were used for analysis of binary response variables. A multivariable analysis of Pindone poisoning could not be performed because the use of this control method was too infrequent.

Ordinal logistic regression was used for the analysis of shooting. The proportional odds assumption was tested according to Hosmer and Lemeshow (2000). Models were developed by backward elimination using the likelihood ratio test to compare the full

model with a reduced model and thus assess the significance of the variables removed ($P < 0.05$). Any variables not originally selected in the univariable screening were then added back into the model and model improvements evaluated based on the likelihood ratio test. The fit of all logistic regression models was tested and logistic regression diagnostics were applied (Hosmer and Lemeshow, 2000). Appropriate Stata commands for ordinal and binary logistic regression accounting for sampling weights, strata and clustering were applied to the final models to calculate odds ratios (OR) and their confidence intervals (Stata Corporation, 2001).

Results

Response rate

The overall response rate for the survey (Table 27) was 71.6% (593 of 828 farmers selected), and the usable response rate was 69.3% (574 responses). Response rates did not differ significantly by region ($P = 0.28$). The response rate by region was 81% for Marlborough, 73% for Manawatu, Southland and Otago, 71% for Wairarapa-Wellington, 70% for Canterbury, 68% for Waikato, and 64% for Hawke's Bay. The response rate from sheep (76%) and mixed sheep-and-beef farmers (77%) tended to be higher than from other farm types (dairy, non-livestock and 'other livestock' farmers: 69%; beef farmers: 67%). A response rate of 84% was achieved from farmers in extreme rabbit-prone areas, compared with 62%, 72%, 70% and 70%, from farms in high, medium, low and negligible proneness categories respectively. The response rate in the high proneness category was relatively low with only about 50% of all farm types responding.

Perceptions of problems caused by rabbits

The proportion of farmers who perceived rabbits to be a problem did not differ between rabbit-proneness categories ($P = 0.84$). Only 21.3% of respondents perceived that they had a rabbit problem on their farms, and 5.7% were uncertain (Table 28). The proportion of farmers perceiving a problem with rabbits was highest in areas of high rabbit-proneness (30.4%), while uncertainty with respect to existence of a problem was most common in areas of extreme rabbit-proneness. Surprisingly, the percentage of

farmers who considered rabbits to be a problem was very similar between the extreme proneness areas and areas of negligible or low proneness.

Table 28: Percentage of farmers who perceived rabbits to be a problem, classified by rabbit-proneness, region and farm type

		Yes	No	Don't know	<i>n</i>
Rabbit-proneness	Negligible	18.3	77.1	4.6	109
	Low	19.6	74.9	5.5	219
	Medium	23.8	69.8	6.4	202
	High	30.4	65.2	4.3	23
	Extreme	19.0	71.4	9.5	21
Region	Canterbury	19.3	74.7	6.0	83
	Hawke's Bay	11.5	78.7	9.8	61
	Manawatu	37.5	56.9	5.6	72
	Marlborough	14.3	83.1	2.6	77
	Otago	18.8	74.0	7.3	96
	Southland	14.8	79.6	5.6	54
	Waikato	28.1	65.6	6.3	64
	Wairarapa	25.4	71.6	3.0	67
Farm type	Beef	18.9	77.4	3.8	106
	Dairy	20.0	76.0	4.0	50
	Non-livestock	26.2	66.4	7.5	107
	Sheep	23.6	69.9	6.5	123
	Sheep-and-beef	13.2	81.4	5.4	129
	Other livestock	30.5	62.7	6.8	59

Although the proportion of farmers who perceived rabbits to be a problem on their farm did not differ between regions (Table 28; $P = 0.23$), this tended to be different for farmers in the North Island compared to the South Island (26.1% vs. 17.1%, respectively; $P = 0.09$).

The proportion of farmers reporting a perceived rabbit problem differed between farm type categories ($P = 0.015$) with the greatest being expressed by farmers of the

'other livestock', non-livestock, and sheep categories, and the least concern being expressed by mixed sheep-and-beef farmers (Table 28).

Farmers considered damage to the soil surface to be the predominant problem attributable to rabbits (Table 29).

Table 29: Farmers perceptions (%) of mechanisms by which rabbits impact on their enterprises (n=574) for each problem caused by rabbits

Main problems caused by rabbits	Severity of rabbit damage					
	None	Minimal	Moderate	Severe	Heavy	Don't know
Compete with livestock for grazing	31.5	38.5	8.9	4.0	1.4	15.7
Damage pasture quality	36.4	29.4	10.3	3.3	1.0	19.5
Destroy vegetables, flowers, grapes, forest seedling etc.	44.1	23.3	10.1	4.2	2.3	16.0
Damage soil surface	23.3	42.9	16.6	5.1	2.8	9.4

On the same farms, the severity of rabbit damage was perceived to be lower in 2001 than in 1995 ($P < 0.0001$), with a shift in rabbit damage from higher ranks of severity to lower ranks (Figure 29).

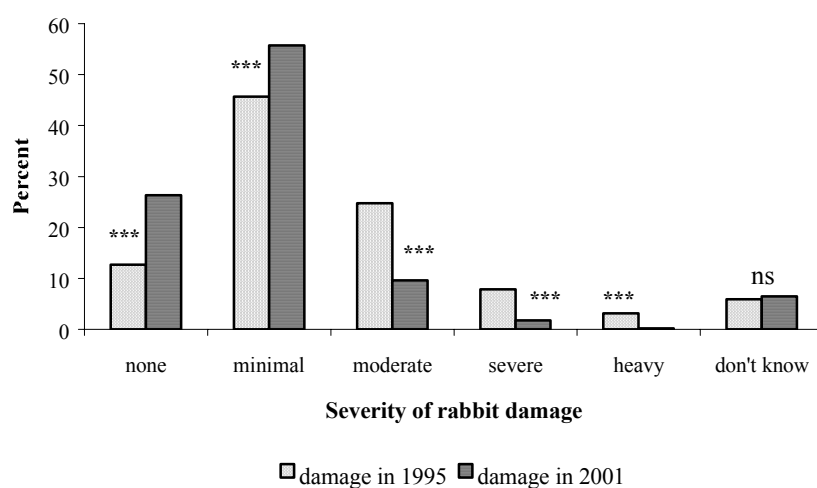


Figure 29: Percentage of farms categorised by rabbit damage severity in 1995 and 2001 ($***P < 0.0001$; ns non-significant $P > 0.10$)

Methods used for rabbit control

The relative frequency of the use of control methods (shooting, 1080 and Pindone poisoning, trapping and RHDV) changed between 1995 and 2001 (Table 30). The proportion of farmers that never used shooting for rabbit control remained constant, but there was a drastic decline in farmers shooting rabbits frequently ('often' and 'very often') from 1995 to 2001. Similarly, the use of 1080 and Pindone poisoning, as well as trapping had decreased to less than half. RHDV was used by 10% of farmers to control rabbits in 2001, but the vast majority of these farmers (90%) used the virus relatively infrequently ('rarely' or 'occasionally').

Table 30: Reported percentage usage of rabbit control methods in 1995 and in 2001 amongst 574 farmers (** $P < 0.01$; ns non-significant $P > 0.10$)

Frequency	Shooting		1080 poisoning		Pindone		Trapping		RHD baiting
	1995	2001	1995	2001	1995	2001	1995	2001	2001
Never	15.3	15.2 ns	82.1	91.3 **	82.9	89.2 **	84.0	89.0 **	89.9
Rarely	8.4	20.0 **	5.9	5.2 ns	5.6	5.2 ns	8.5	7.1 ns	5.1
Occasionally	38.3	45.3 **	6.8	3.0 **	8.2	4.9 **	6.3	3.1 **	4.0
Often	26.8	14.8 **	3.8	0.4 **	2.3	0.7 **	1.0	0.5 ns	0.7
Very often	11.1	4.7 **	1.6	0.2 **	1.0	0.0 **	0.2	0.2 ns	0.3

Shooting

The frequency of shooting was similar in all regions but differed between farm types ($P = 0.015$) and rabbit-proneness categories ($P = 0.046$). Most respondents indicated that they would 'occasionally' shoot rabbits. A relatively high proportion of 'other livestock' farmers (34%) often shot rabbits while a substantial fraction (25%) of 'non-livestock' farmers would never shoot rabbits. Sheep as well as mixed sheep-and-beef farmers increasingly used shooting for rabbit control as rabbit-proneness increased; almost half of all sheep farmers shot rabbits sometimes or often when rabbit-proneness was medium to severe. In contrast beef and dairy farmers rarely controlled rabbits by means of shooting, regardless of rabbit-proneness. However, there were very few beef or dairy farmers in the survey that had high or extreme rabbit-prone properties.

RHD baiting

The percentage of farmers using RHDV baiting differed between regions ($P = 0.01$). RHDV baits were most frequently used in Canterbury (18.1%) and Otago (15.6%), while only 3-10.4% of farmers used this control method in other areas. RHD use was positively associated with rabbit-proneness ($P < 0.001$), increasing from less than 10% of farms in negligible and low rabbit-prone areas to almost half of farms in extreme rabbit-prone areas (Table 31). However, RHDV use did not differ between farm types ($P = 0.74$).

Table 31: Percentage of farmers using RHDV in 2001 stratified by rabbit-proneness

Rabbit-proneness	RHD baiting
Negligible	5.5
Low	6.9
Medium	11.4
High	17.4
Extreme	47.6

The proportion of farmers using RHD baiting stratified by farm type and rabbit-proneness varied significantly (Figure 30; $P = 0.04$). Mixed sheep-and-beef farmers, non-livestock and sheep farmers in extreme rabbit-prone areas were the largest users of RHD baiting (63%, 50% and 43% respectively). 'Other livestock' farmers were most likely to use RHD in high rabbit-prone areas (50%) and dairy farmers in medium rabbit-prone areas (23%).

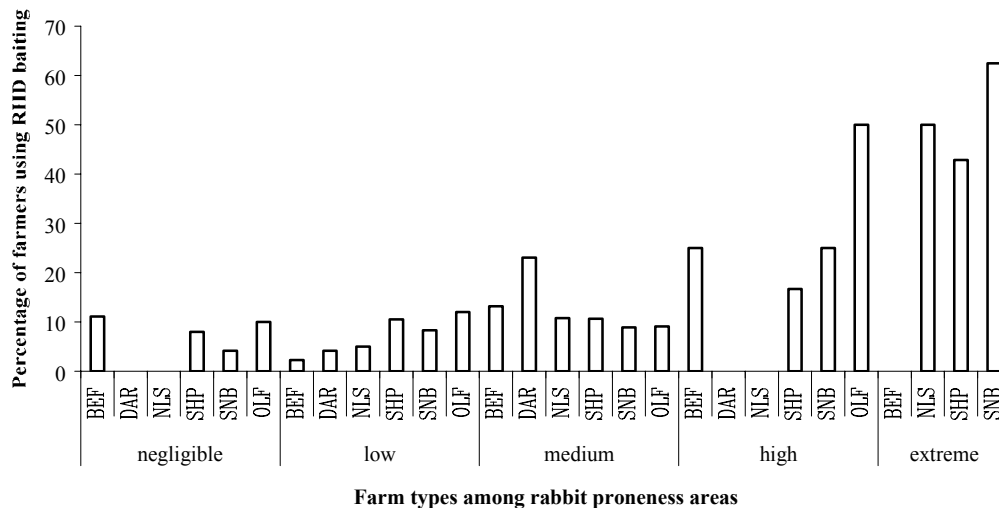


Figure 30: Use of RHD baiting by 58 farms for different farm types (BEF beef, DAR dairy, NLS non-livestock, SHP sheep, SNB mixed sheep-and-beef, OLF other livestock) in five different rabbit-prone areas in 2001 (as percentages)

Use of poisoning and trapping

Prevalence of use of 1080 poisoning for rabbit control did not differ between regions ($P = 0.08$) or farm type ($P = 0.32$). However, it did differ between rabbit-proneness categories ($P < 0.001$), with the highest use of 1080 poisoning occurring in the extreme rabbit-prone areas (33%). The interaction of farm types with rabbit-proneness categories was not significant ($P = 0.63$).

In contrast, Pindone use was dissimilar between regions ($P = 0.045$), but not between farm types ($P = 0.24$) or proneness categories ($P = 0.13$). Farmers in the Waikato (19%) and Canterbury (16%) regions were most likely to use Pindone, while only 0.5% of farmers in Hawke's Bay and 5% in Southland used Pindone.

The overall percentage of farmers who trapped rabbits was 11%, and did not differ between regions ($P = 0.61$), farm type ($P = 0.89$) or rabbit-proneness category ($P = 0.24$).

Farmers' perceptions about rabbit control methods

Figure 31 shows the distribution of farmers' perceptions about the humaneness of these control methods among respondents who recorded an opinion other than uncertain. Shooting was viewed to be the most humane method, followed by RHD, Pindone and 1080 poisoning, myxomatosis infection, and trapping. While opinions of the humaneness of the two poisoning methods were very similar, opinions differed markedly between the two biological agents. For RHDV, 82% of respondents found the method satisfactory, humane or completely humane compared to only 44% for myxomatosis.

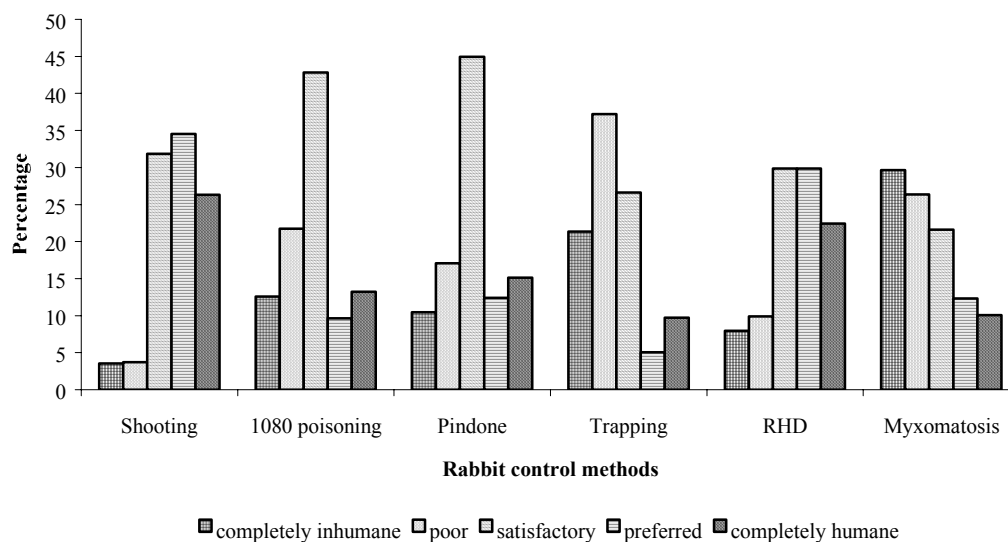


Figure 31: Frequency distributions of farmer opinion about the humaneness of rabbit control methods

The percentage of farmers that were uncertain ('don't know') about the humaneness of rabbit control methods was only 2% for shooting, compared with 18% for trapping, 22% for 1080, 28% and 31% for RHD and myxomatosis respectively, and 55% for Pindone poisoning.

With respect to environmental safety, ethical concerns were higher for poisoning (particularly 1080) than for infectious agents, and lowest for shooting and trapping (Figure 32). Many respondents were uncertain about the impact of Pindone poisoning (54%) and the infectious agents (RHDV 35%; myxomatosis 44%) on the environment.

In contrast, only a few respondents were uncertain about shooting (3%), trapping (13%), and 1080 poisoning (16%).

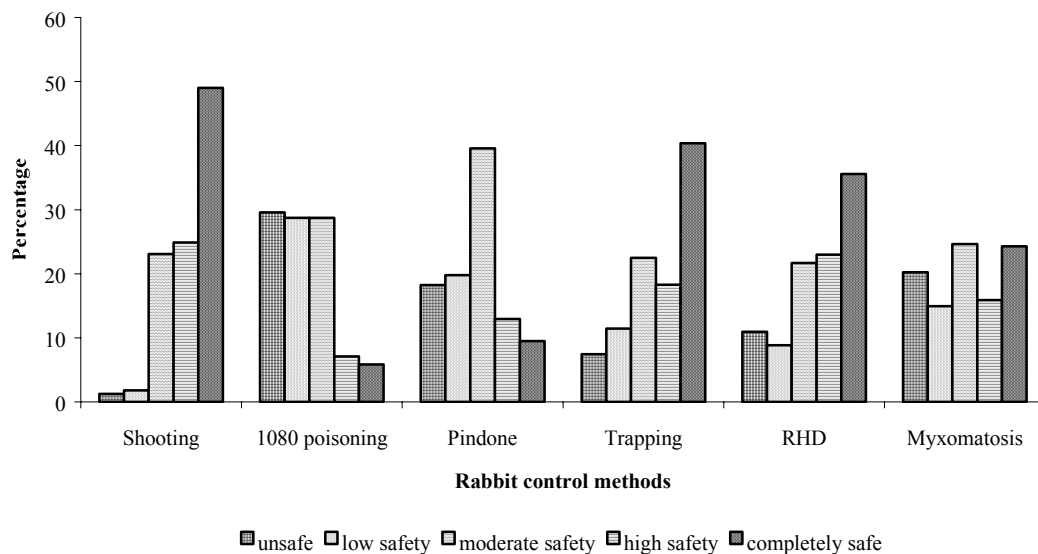


Figure 32: Frequency distributions of farmer opinion about the environmental safety of rabbit control methods

Farmers who recorded an opinion on the efficacy of rabbit control methods (Figure 33) considered RHDV to be by far the most effective method (78% high or very high), followed by 1080 poisoning (60%) and shooting (35%). Only 11% of respondents considered the efficacy of trapping to be high or very high. The responses regarding the efficacy of Pindone poisoning (24% high or very high) were difficult to interpret, due to the large proportion of farmers (61%) who appeared to be unfamiliar with the effectiveness of this agent. High levels of uncertainty about effectiveness were also expressed about RHDV, 1080 and trapping (30%, 29%, and 23% respectively), while only 3% had no opinion about the effectiveness of shooting.

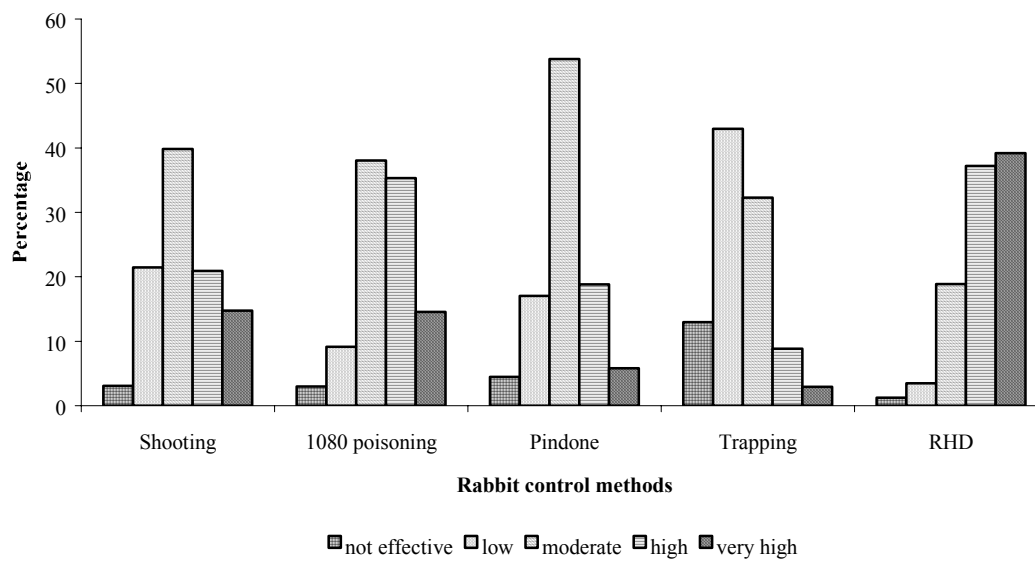


Figure 33: Frequency distributions of farmer opinion about the effectiveness of rabbit control methods

Factors associated with the use of rabbit control methods

Shooting

The final multivariable ordinal logistic regression model included the categorical variables 'compete with livestock for grazing' and 'effectiveness of shooting' (574 farms, 10 strata, 28 groups of rabbit proneness multiplied by farm type, $P < 0.0001$). The use of shooting for rabbit control was strongly associated with the perception of the extent to which rabbits 'competed with livestock for grazing', and were markedly elevated among groups rating the level of competition as severe or heavy (Figure 34).

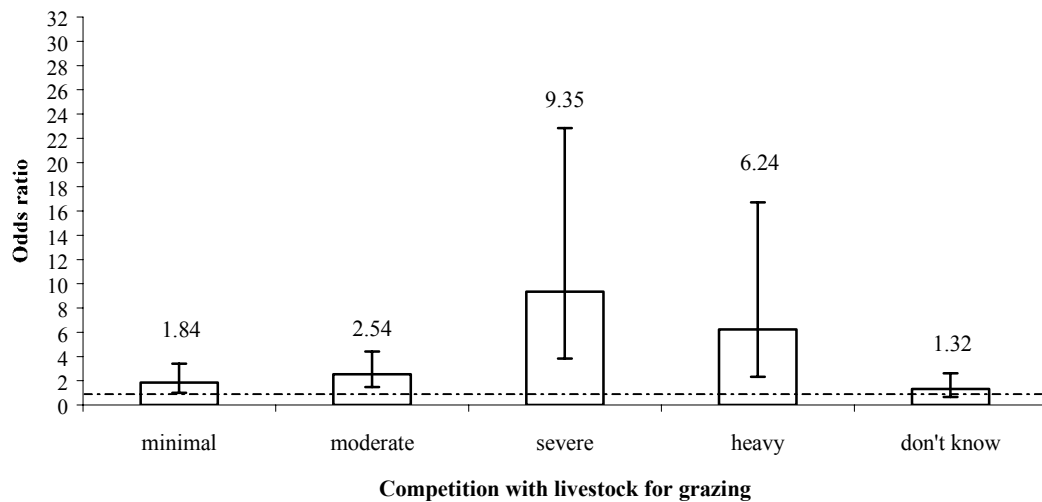


Figure 34: Odds ratio (95% CI) describing the strength of the association between shooting and farmers' perceptions on the extent that rabbits compete with livestock for grazing (reference group: 'no competition with livestock for grazing,' dotted line illustrates Odds Ratio of 1)

The association between the perceived 'effectiveness of shooting' and the odds of shooting was not statistically significant. However, the data indicated a 'dose response' relationship where the odds of shooting increased with the perception that shooting was effective (Figure 35).

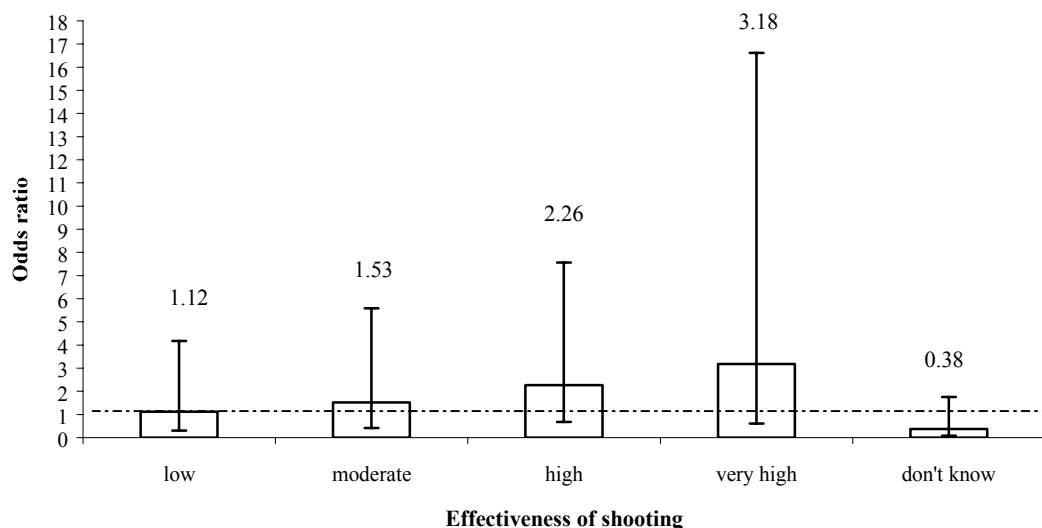


Figure 35: Odds ratio (95% CI) describing the strength of association between shooting and farmers' perceptions on the effectiveness of shooting (reference group: 'not effective,' dotted line illustrates Odds Ratio of 1)

Use of other control methods

The final multivariable binary logistic regression model for the odds of RHDV use is shown in Table 32. As with shooting, the odds for RHDV use were significantly associated with the perception of the extent of rabbits competing for grazing. Although the odds for the use of RHDV were not significantly related to the perception of humaneness, there was a trend towards the more likely use of RHD as the perception of humaneness increased.

Table 32: Odds ratios (OR) from binary logistic regression of the use of RHDV to control rabbits (574 farms, 28 groups of proneness multiplied by farm type)

Explanatory variable	Categories	OR	OR 95% CI
Compete with livestock for grazing	None	1.00	
	Minimal	5.09	1.35 - 19.15
	Moderate	5.86	1.20 - 28.62
	Severe	15.74	3.57 - 69.37
	Heavy	59.48	9.63 - 367.55
	Don't know	2.81	0.67 - 11.80
Humaneness of RHD	Completely inhumane	1.00	
	Poor	0.71	0.03 - 17.87
	Satisfactory	1.09	0.16 - 7.38
	Preferred	1.84	0.31 - 10.91
	Completely humane	1.56	0.19 - 13.08
	Don't know	0.04	0.00 - 0.67

The final logistic model for the odds of farmers using trapping included the variables of damage to pasture quality, effectiveness of trapping, and humaneness of myxomatosis. Use of trapping was strongly associated with the perceived effectiveness of trapping, and also with the perception of the level of pasture damage caused by rabbits. The odds of use of trapping were also higher for farmers who perceived that myxomatosis was inhumane or were uncertain about its humaneness.

The use of 1080 poisoning for rabbit control strongly increased with both the extent to which farmers perceived rabbits to compete for grazing and with the perceived safety of 1080.

Farmers' concerns about Rabbit Haemorrhagic Disease

Depending on the region and level of rabbit-proneness but independent of farm type, a variable proportion of respondents had seen rabbits dying from RHD on their properties. The proportion of farmers observing rabbit deaths due to RHD increased with rabbit-proneness. The highest proportions of farmers witnessing the disease were from Otago (43%) and Canterbury (41%). Hawke's Bay had the lowest proportion of farmers witnessing the disease (10%), which is consistent with the fact that a high number of respondents (28%) were uncertain whether rabbits had died from RHD in the area.

The concerns of farmers about a range of questions regarding the success, worries and future directions of RHDV for rabbit control are summarised in Table 33. Many statements expressed considerable uncertainty. A majority of farmers agreed that the use of RHD had reduced rabbit numbers (65%), considered RHD to be a cost-effective method (57%), and thought that it was generally beneficial (64%). There was great uncertainty among respondents about the question of ecological risks of RHDV use in New Zealand (44%). However, 35% of respondents disagreed that the virus is an ecological hazard, and 35% would support the introduction of other diseases to kill rabbits. More than half of the respondents would like to see more research into RHD (56%) and considered that RHDV is best used in conjunction with other methods (56%).

Table 33: Farmers' opinions on aspects of RHDV as a tool for rabbit control (tables shows percent of 574 respondents)

	Strongly disagree	Disagree	Don't agree or disagree	Agree	Strongly agree	Don't know
RHD brought rabbit numbers down	1.6	3.3	3.7	27.9	37.1	26.5
RHD is still working very well	2.6	10.1	11.1	22.6	12.4	41.1
RHD was only a short-term solution and is not working as well now	9.1	18.1	12.2	17.2	2.4	40.9
RHD is more cost effective than other methods	0.5	1.6	5.1	28.7	28.0	36.1
RHD should be used with other methods	2.4	4.2	6.6	39.4	16.9	30.5
The introduction of RHD had no benefits	30.8	33.1	4.5	2.6	1.7	27.2
RHD is a danger for the ecology of New Zealand	16.7	18.3	11.1	5.7	4.2	43.9
More research is needed to make RHD fully effective	3.1	5.7	8.4	42.0	14.3	26.5
Other diseases should be introduced to kill rabbits	15.0	19.7	14.3	11.7	5.9	33.4

Discussion

New Zealand enjoys trading advantages for its animal products due to the country's absence of many important livestock diseases, and allocates considerable resources to biosecurity measures aimed at preserving the health status of its national herds. The deliberate release of RHDV in New Zealand, subsequent to governmental refusal to introduce the virus, was not a malicious 'bioterrorist' action, but an action motivated by perceptions that RHDV would provide an option for rabbit control that was superior to pre-existing methods. The results of this survey provide some insights into the attitudes and practices of farmers in the wake of several years of experience following the release of RHDV, and farmers' perceptions on the impact of the disease in New Zealand.

In all countries, agricultural enterprises are diverse and opinions of farmers can be expected to be highly variable within and between regions and industries. Our objective in undertaking this study was to obtain unbiased estimates of opinions of farmers engaged in key agricultural industries in New Zealand, with particular emphasis given to pastoral livestock producers. The availability of a national spatially-referenced farm database (Agribase) and geographical data on rabbit-proneness underpinned the strategy to stratify sampling according to region, rabbit-proneness and enterprise type. Random sampling of farms within strata should have minimised selection bias, and the useable response rate (69%) should have been sufficient to avoid marked response bias. However, the study population was limited to eight regions encompassing approximately 63% of the eligible farm population, and some regional bias is possible. The matrix of sampling weights was used to enable more precise estimates for sub-groups of greatest interest, and the weightings were accounted for in the analysis. A major limitation of our approach was the aggregation of all non-livestock enterprises into a single group, and it is acknowledged that considerable variability in farmer opinions could exist between the diverse enterprise types in this group. Similarly, the aggregation of fundamentally different enterprises such as horse and poultry production within the 'other livestock' category may have masked quite different perceptions existing among sub-groups of this stratum.

With respect to the magnitude of the rabbit problem in New Zealand, only 21% of farmers considered rabbits to be a problem on their farms in 2001, and the proportion of respondents reporting moderate to heavy rabbit damage declined by approximately two thirds between 1995 to 2001. The somewhat unexpected finding that the proportion of farmers reporting that rabbits were a problem in 2001 did not vary significantly with rabbit-proneness, may in part be attributable to the fact that the farms in the most affected areas have been able to reduce the severity of the problem. The data strongly support the conclusion that rabbit infestation in extreme rabbit-prone areas was reduced after introduction of RHD (Parkes et al., 1999). Enterprise type was associated with the farmers' perception of having a rabbit problem, with concern most prevalent among the diverse group of 'other livestock' farmers. Damage of the soil surface (which is probably the most readily apparent form of damage) was perceived to be the most important mechanism of rabbit damage. One aspect of soil damage is the occurrence of holes and collapsing burrows, which increases the risk for lameness and foot problems in

livestock and horses. Lameness as a result of rabbit burrowing was one of the most frequent additional comments made by 'other livestock' farmers.

The release of RHDV in 1997 has been associated with altered patterns of rabbit control by farmers between 1995 and 2001. Although only 10% of farmers used RHDV baits in 2001, some reduction was evident in the use of all pre-existing control methods. Thus the perceived reduction in the magnitude of rabbit problems has occurred during a period when the reported use of all traditional control methods has declined, and is likely to be a consequence of the impact of RHDV. However it is clear that RHDV has not been a quick fix for the rabbit problem as all pre-existing methods continued to be employed by farmers, with shooting remaining the most prevalent method. While respondents generally thought that RHD had resulted in reduced rabbit numbers, the predominant opinion was that RHD was best used in conjunction with other methods.

Several patterns emerged with respect to the selection of methods used for rabbit control. RHD baiting was most frequently used in the extreme rabbit-prone areas of the South Island, particularly on farms associated with sheep and on non-livestock farms. The poison 1080 was used more frequently in extreme rabbit-prone areas, and Pindone use was highest in the Waikato region. Only trapping was conducted evenly across regions, farm types and rabbit-proneness strata. Clearly, the choice of methods for rabbit control will be subject to diverse influences including farmer's personal perceptions of efficacy and humaneness, as well as local customs, marketing and peer-group influences. Evaluation of associations between a range of explanatory variables and the use of respective control methods identified some likely factors. Generally use of rabbit control methods was associated with perceived severity of rabbit damage (competition with livestock for pasture and degree of damage to pasture quality) and perceived attributes of the method (e.g. humaneness and efficacy of a respective method).

Opposition to the release of RHDV was based on environmental safety and animal welfare concerns. It is notable that respondents ranked RHD to be the second most humane method after shooting, and preferable to poisoning. Also, while farmer perceptions of the humaneness of the two poisons were almost identical, perceptions regarding the two viral agents were very different. Myxomatosis was unsuccessfully

introduced into New Zealand (Filmer, 1953). In contrast to the peracute deaths typical of RHD, wild rabbits infected with the *Myxoma virus* succumb slowly (within 10-14 days) to the disease with symptoms of exhaustion, anorexia, depression, laboured breathing and pneumonia (Lutz, 1990). Trapping of rabbits, considered inhumane by 21% of respondents, is usually conducted with leg-hole traps and rabbits remain in the traps until released. The level of concern about the humaneness of trapping is consistent with a recommendation to ban trapping due to animal welfare concerns (Williams et al., 1995).

Safety concerns of farmers were greatest for the poisoning methods, particularly 1080 (30% classified it as unsafe), that has been used for rabbit control since the 1950s (McIntosh, 1958). It is highly toxic for all mammals with carnivores being more susceptible than rabbits, and has no antidote (Williams et al., 1995). Despite rapid elimination of the poison from animals that consume sub-lethal doses (Eason, 1992), secondary poisoning has been reported in rabbit predators (Heyward and Norbury, 1999). Negative opinions from farmers about 1080 are probably driven by the experiences of death of farm dogs and domestic livestock and as well the current public debate on killing deer by aerial baiting (Hansford, 2003). In a 1996 survey of the New Zealand public, Wilkinson and Fitzgerald (1998) found that 28% of respondents considered 1080 poison the least safe method (28% high risk), while 22% of the respondents considered RHDV to have a high safety risk. A notable point of our survey is that 10% of farmers continue to have reservations about the ecological safety of RHDV despite a lack of any documented safety related incidents since the release of the agent. New Zealanders have high levels of concern about the environment (Macer, 1994), and the possibility that predation of native bird species may be an unwanted consequence of improved rabbit control has been proposed (Haselmayer and Jamieson, 2001).

Assessment of the efficacy of rabbit control methods is not straightforward, and is reflected in the considerable proportion (20-60%) of respondents who were uncertain about the efficacy of all methods other than shooting (3%). Field observations indicate that the impact of RHD on wild rabbit populations can be highly variable (Mutze et al., 2002; Parkes et al., 2002). However, 39% of respondents ranked RHD highest for efficacy, which was 2-3 fold higher than the next highest ranked method.

Based on a survey conducted before RHDV release, Wilkinson and Fitzgerald (1998) reported that half of the respondents believed that RHD would bring more benefits to New Zealand than problems. Similarly, the majority of farmers (approximately 60%) in our survey stated that the introduction of RHDV has been beneficial overall. However there were clear indications that the introduction of the agent has been far from a complete solution to the rabbit problem, and many respondents expressed uncertainty about most aspects of the disease and its impact.

We conclude that farmers perceive the use of RHDV for rabbit control to be an effective and ethically acceptable method. Since its introduction, the use of shooting has declined and other methods are regarded as less effective or less acceptable than RHD.

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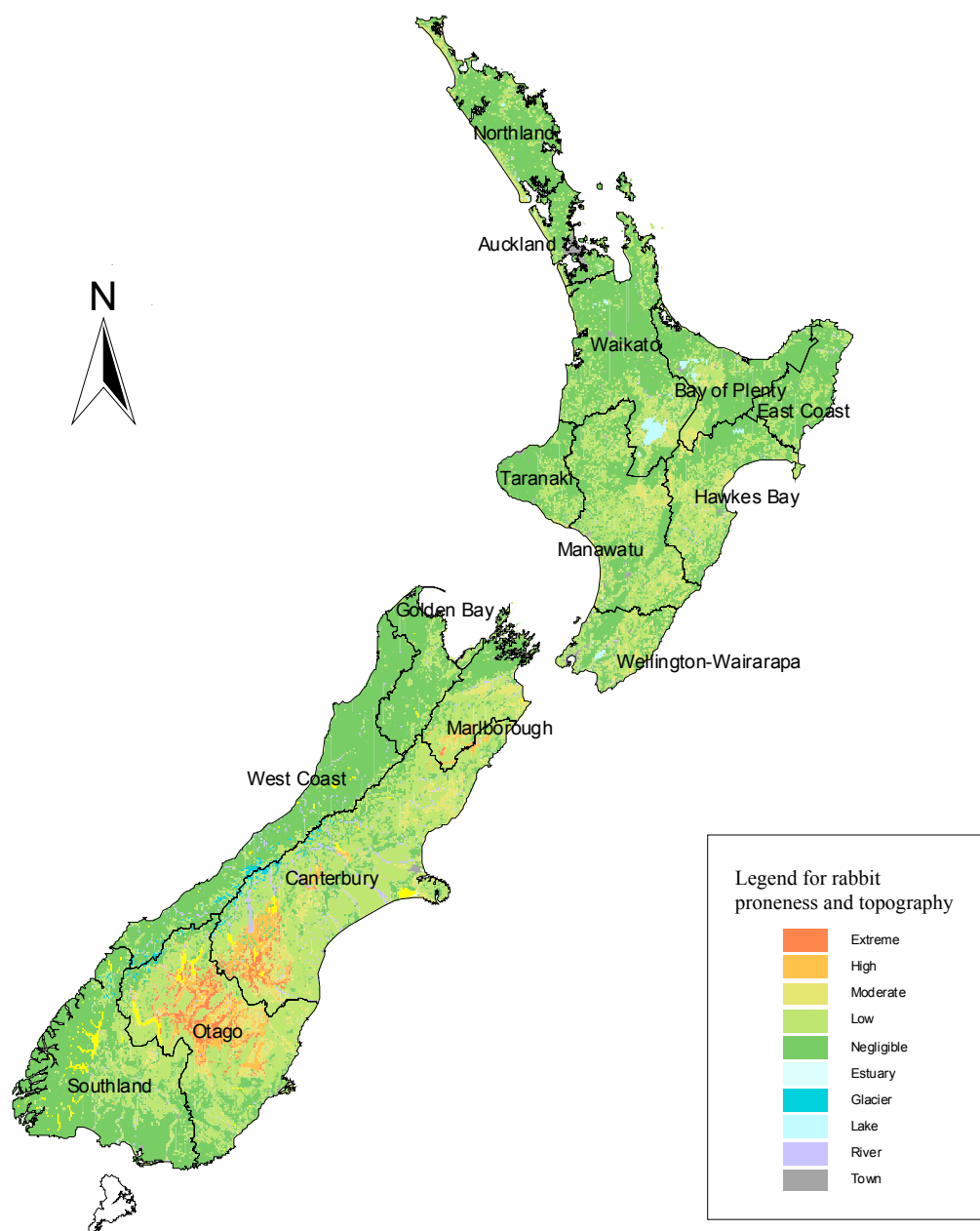
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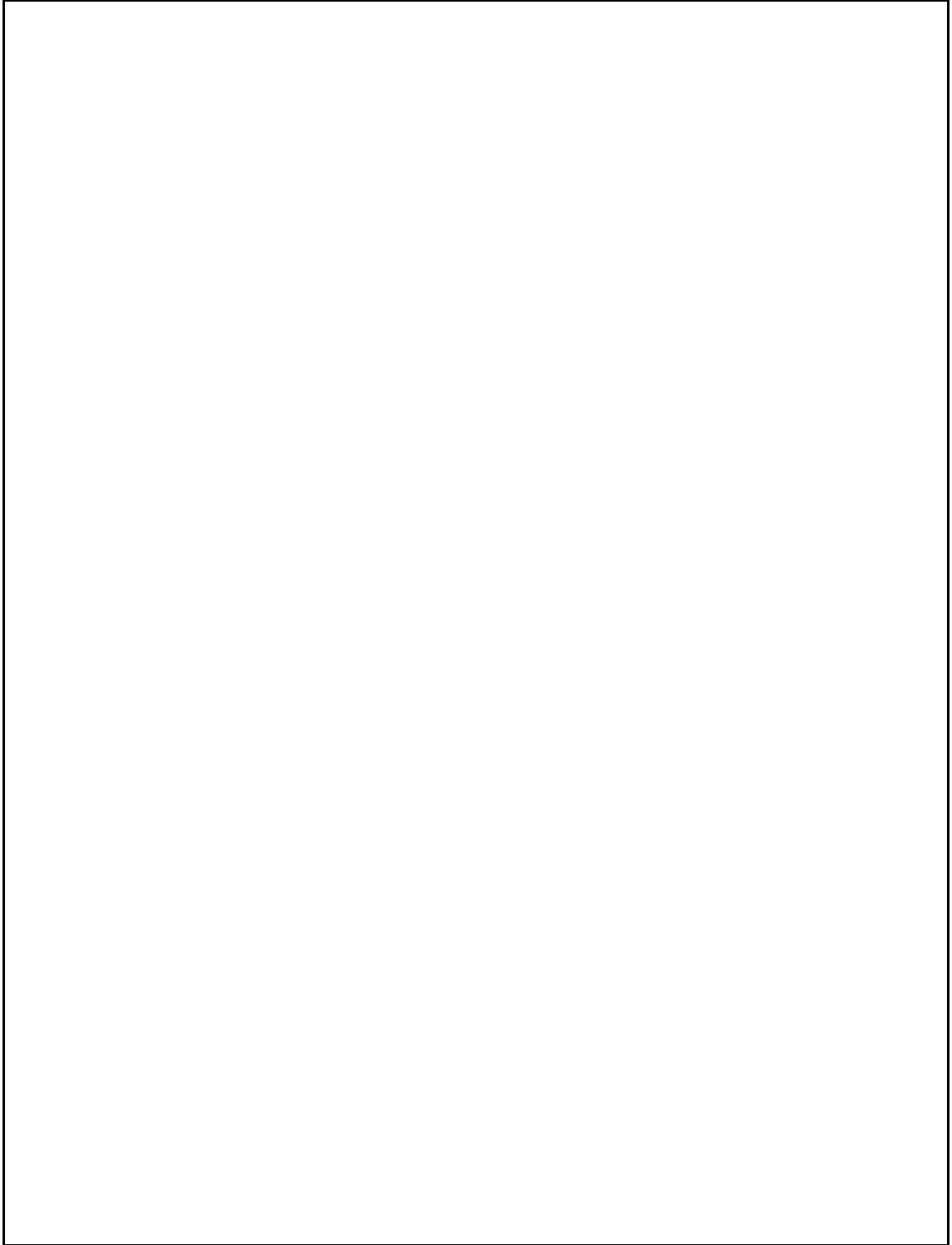
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Appendix 3: Rabbit-proneness map of New Zealand used as a primary spatial stratum for selection of farms. Selected farmers were surveyed on methods used to control wild rabbits (described in chapter 8)





6. How do you rate the effectiveness of the following methods to kill rabbits?
Please tick one box per row.

	not effective	low	moderate	high	very high	don't know
Shooting	☐	☐	☐	☐	☐	☐
1080 poisoning	☐	☐	☐	☐	☐	☐
Pindone	☐	☐	☐	☐	☐	☐
Trapping	☐	☐	☐	☐	☐	☐
RHD	☐	☐	☐	☐	☐	☐

7. How often did you use the following control methods in 1995? Please tick one box per row.

	never	rarely	occasionally	often	very often
Shooting	☐	☐	☐	☐	☐
1080 poisoning	☐	☐	☐	☐	☐
Pindone	☐	☐	☐	☐	☐
Trapping	☐	☐	☐	☐	☐

8. How often do you **currently** use the following control methods? Please tick one box per row.

	never	rarely	occasionally	often	very often
Shooting	☐	☐	☐	☐	☐
1080 poisoning	☐	☐	☐	☐	☐
Pindone	☐	☐	☐	☐	☐
Trapping	☐	☐	☐	☐	☐
RHD baiting	☐	☐	☐	☐	☐

RHD

9. Have you ever seen rabbits dying from RHD on your property?
yes ☐ no ☐ don't know ☐

10. When have you seen rabbits dying from RHD on your property? Please indicate the month and the year and tick the appropriate box for the type of RHD occurrence.

number of outbreaks	month	year	natural RHD outbreak	spread by RHD baiting
1.			☐	☐
2.			☐	☐
3.			☐	☐
4.			☐	☐

11. Why do you think those rabbits died from RHD?

12. What do you think about the following statements related to RHD in your area? *Please tick one box per row.*

	strongly disagree	disagree	don't agree or disagree	agree	strongly agree	don't know
RHD brought rabbit numbers down	☐	☐	☐	☐	☐	☐
RHD is still working very well	☐	☐	☐	☐	☐	☐
RHD was only a short-term solution and is not working as well now	☐	☐	☐	☐	☐	☐
RHD is more cost effective than other methods	☐	☐	☐	☐	☐	☐
RHD should be used with other methods	☐	☐	☐	☐	☐	☐
The introduction of RHD had no benefits	☐	☐	☐	☐	☐	☐
RHD is a danger for the ecology in New Zealand	☐	☐	☐	☐	☐	☐
More research is needed to make RHD fully effective	☐	☐	☐	☐	☐	☐
Other diseases should be introduced to kill rabbits	☐	☐	☐	☐	☐	☐

General information

13. Which of the following age groups do you belong to? *Please tick the appropriate box.*

Under 20	20-29	30-39	40-49	50-59	60 or over
☐	☐	☐	☐	☐	☐

14. What is your gender?

female ☐ male ☐

15. What is the your farm type? *Please tick one box*

Beef cattle farming	Dairy cattle farming	Sheep farming	Mixed sheep/beef farming	Other livestock	Horticulture (vegetables, grapes, fruits, flowers etc)
☐	☐	☐	☐	☐	☐

Chapter 9

General Discussion

RHDV in wild rabbit populations

There are numerous examples of negative unintended consequences from introducing exotic species into new environments. Therefore any proposal to introduce an apparently novel animal pathogen, such as RHDV, into New Zealand, a country with an island ecosystem and an economy that is highly dependent on animal agriculture, will inevitably be subject to considerable scrutiny and criticism. In 1997, the New Zealand government rejected an application by farmer groups to introduce RHDV as a biological control method for rabbits, because of uncertainties about the likely success of RHDV in the New Zealand environment and the ecological risks (O'Hara 1997). These concerns were amplified by a general paucity of knowledge about the epidemiology of the agent and the disease. Subsequently, the illegal introduction of RHDV, and the resulting disease outbreaks, provided a rare opportunity to study a disease soon after its initial occurrence in a region. The intensive sampling conducted in the longitudinal studies have provided detailed data on the temporal and spatial aspects of the disease over a period of nearly three years. In addition experimental studies revealed properties of RHDV that are important for understanding the disease.

The longitudinal study confirmed that RHDV had become established in the study region and showed evidence of transmission recurring in annual cycles. However, the cause-specific mortality rate varied considerably between years. The magnitude of naturally occurring outbreaks of RHDV is likely to be influenced by population demographics, individual and population immune statuses, competing risks due to other causes of mortality, and climatic conditions that may influence virus survival, vector abundance and rabbit behaviour. Rabbit movements, vector abundance, and activity of predatory or scavenging species probably facilitate the spread of RHDV. Prediction of naturally recurring RHDV outbreaks requires consideration of these factors, which are all likely to influence the success of deliberate RHDV releases to control rabbits.

The source of RHDV infection that initiates any outbreak is usually difficult or impossible to identify, and therefore open to speculation. The importance of predators, scavengers and insects in mechanical transmission of RHDV will in part be determined by the durability of the virus in carcasses. This question is difficult to address because RHD virus is not culturable *in vitro*, and therefore bioassays in live rabbits are required.

The ethical cost of conducting such studies is considerable and the evidence base for evaluating the importance of viral survival outside the host remains minimal. Nevertheless, the studies undertaken clearly indicate that RHDV in a matrix of organic tissue could remain a potential source of new infections for at least three months. In contrast, RHDV on cotton exposed directly to environmental conditions (used to emulate excreted virus on fomites) was less persistent. Thus excreted virus in the environment is likely to play a major role in transmission once an epidemic is initiated, but is less likely to contribute to reservoirs responsible for inter-epidemic survival of the virus. In addition, the failure of inactivated virus to elicit serological responses in rabbits suggests that unintended 'vaccination' of wild rabbit populations with baits is an unlikely event.

The probable existence of carrier rabbits is now generally accepted (Parkes *et al.* 2002; Cooke 2002; Zheng *et al.* 2002), despite the lack of credible supporting data. An important question is to define which sub-group of rabbits is the predominant source of virus. Because the duration of viral shedding in acute and peracute cases is short, due to the rapid mortality, most virus shedding will probably occur in rabbits that have recently survived RHD outbreaks (White *et al.* 2002). However, the mean duration of excretion of infective virus, and its variance, has yet to be quantified. Also it is not known whether excretion of RHDV from infected rabbits is constant or intermittent, nor if intermittent, how frequently it occurs. It is feasible that infected rabbits not excreting virus could provide a source of infection due to predation or scavenging after death from other causes. Mortality of RHDV in susceptible adults is very high compared to other infectious disease in wildlife hosts, such as tuberculosis in possums (Ron Jackson, personal communication), hence relatively few adult survivors remain to spread the disease. Alternatively, young animals may survive infection if maternal immunity or natural resilience is present, and could serve as long-term reservoirs of virus.

Persistent, low-level excretion by chronically infected rabbits, as modelled by White *et al.* (2002), could also be a reservoir of infection. In this scenario, interactions of extrinsic factors trigger increased excretion, leading to spread of the infection. However, reactivation of RHDV in experimentally infected rabbits (4-5 weeks old) which recovered from the disease was not achieved by treatment with dexamethasone or classical swine fever virus vaccine (Shien *et al.* 2000). At this point in time the

possibility of reactivation of shedding by chronically infected rabbits remains hypothetical.

The observation of seropositivity in several non-target species raises questions with respect to both the host range of the virus and the role of such species in the dissemination and transmission of the virus. It remains to be determined whether the serological reactions observed reflect the occurrence of viral replication in non-target species or a simple response to viral antigens. The fact that we were unable to elicit serological responses to UV inactivated RHDV in rabbits following oral and parenteral exposure would suggest that seroconversion in the absence of viral replication is unlikely. However, there is still no convincing evidence confirming that non-target species can excrete RHDV, and this question warrants further investigation. It must also be remembered that serological tests for RHDV have not been validated for non-target species and positive results should be interpreted with caution.

A further outstanding issue in the epidemiology of RHDV in New Zealand is the suggested existence of a benign RHDV-like virus. A non-pathogenic calicivirus has been isolated in Europe. To date no such virus has been identified in either New Zealand or Australia and evidence for such a virus in New Zealand is limited to sparse serological data. In the current studies, there were only three rabbits with serological profiles that were consistent with infection with 'pre-existing' virus, and no indication that these rabbits were protected against RHDV. Recent modelling studies in the United Kingdom concluded that the development of acute and chronic infection states was sufficient to explain variability in the effect of RHDV in the absence of a benign and cross-protecting virus (White *et al.* 2002). In order to understand the reasons behind the apparent temporal and spatial variability in RHD severity, detailed studies of 'successful' and 'unsuccessful' RHD outbreaks will need to be conducted (Cooke 2002; Henning and Davies 2003). Localised patchiness may be influenced by environmental barriers of vegetation, which in turn are correlated with the abundance of flying vectors. In this study we found that RHDV deaths were spatially clustered within the study site, and hot-spots of RHD recurrence can be expected.

Fly vectors

We identified several climate parameters associated with abundance of the fly species considered most likely to play a role in the transmission of RHDV in New Zealand. Climate influences fly activity, ovi- and vivipositions and fly development. It also affects the decomposition of carcasses, which are a source of contamination of flies with RHDV. Disease transmission by flies can either occur directly to healthy rabbits or indirectly through contamination of the environment. Observed differences in the climate pattern and associated fly abundance, between corresponding periods in different years could be one factor contributing to variability in the months in which RHD outbreaks were observed in 2000 and 2001. Previously, climate changes have also been linked with virus survival and rabbit breeding (Henzell *et al.* 2002). However, it is unlikely that occurrence of an outbreak will be dictated by a single factor that is climate dependent. The seasonal pattern of outbreaks in late summer and autumn is most probably a result of the interaction of multiple factors related to the host population and environment, many of which may be influenced by climatic factors.

Given the apparent temporal association between fly abundance and the occurrence of RHD outbreaks, high fly abundance may serve as a marker for environmental conditions that favour RHDV transmission. The release of virus-contaminated baits is probably best suited to warm dry periods of optimal fly breeding and activity. Rabbits succumbing to RHD should be left on pasture close to ground cover, where they are easily accessed by fly species. As previously reported by Barratt *et al.* (1998), the role of *Oxysarcodexia varia* in particular could be enhanced by stock movements to provide dung-breeding opportunities for this species.

Methods of rabbit control in relation to natural mortality

Rabbit numbers and rabbit damage were reduced following RHDV introduction and the overall effect of RHDV is generally considered satisfactory by farmers. Some of the more conventional control methods have limitations, in particular poisoning. Use of 1080 over several decades resulted in bait avoidance (neophobia). Neophobia, an avoidance of novel objects, is particularly prevalent in areas of long-term poisoning (Rowley 1963; Oliver *et al.* 1982). A second type of aversion is bait shyness, an

avoidance of toxic baits through negative conditioning to bait related effects (Poole 1963; Devine and Cook 1998). Furthermore there is a risk of poisoning non-target species (Booth and Wickstrom 1999; Powlesland *et al.* 2000; Lloyd and McQueen 2002). In addition to improving the overall effectiveness of rabbit control, the introduction of RHDV has reduced the environmental and administrative problems associated with 1080 poisoning, as well as the animal welfare concerns related to trapping. Consistent with an analysis conducted in Australia by Saunders *et al.* (2002), this should have reduced the overall cost of rabbit management, and thus increased the economic viability of properties previously afflicted by high rabbit numbers.

RHDV will not replace shooting as the most popular rabbit control method. Barlow (1999) described a rabbit density-dependent model in which a supplementary control method like shooting may have adverse consequences for RHD effectiveness if rabbit density is reduced below the threshold level for disease persistence. However, the number of remaining rabbits depends on initial rabbit density, and we have shown that RHD can also recur annually in sparse rabbit populations under influence of shooting (Henning and Davies 2003). The impact of natural RHD outbreaks can differ between years, and shooting may be a preferred option in years with low RHD mortality. Parkes *et al.* (2001) also suggested a change from the expensive active employment of conventional control methods (aimed to maintain constantly low rabbit densities) to a cheaper, more reactive strategy of applying conventional control methods only when RHD was not successful. Active RHDV ‘biociding’ is mainly practised in the most rabbit prone areas. Investigation of the causes of success and failure of RHDV releases was not an objective of the research presented in this thesis, but remains an important issue for optimisation of biological control of rabbits with RHD (Henning, unpublished data). Likely factors include rabbit population biology and immunity status, virus survival and vector activity (Bowen and Read 1998; Parkes *et al.* 1999).

The interactions between natural RHD outbreaks, biociding and shooting occur in a broader context that includes other competing causes of rabbit death, and in particular predation. There is debate over whether predators can regulate rabbit populations (Newsome *et al.* 1989; Krebs 1995; Sinclair and Pech 1996). Some authors consider predation as a limiting, but not regulating, factor (Banks 2000) while others have shown predator regulation of rabbits especially in low rabbit density areas (Pech *et al.* 1992).

Reddiex *et al.* (2002) demonstrated that RHD has a higher impact when predators are not controlled. Predation was the principal cause of mortality in our study and resulted in more consistent rabbit mortality compared to the more dramatic, but transient short term impact of RHD epidemics.

Active rabbit control can be viewed as the deliberate augmentation of natural causes of death, which are widespread and prominent, with additional risks of mortality. Natural mortality will fluctuate over time, as will rabbit reproductive efficiency, and human intervention is an appropriate complementary tool for population regulation in times of high rabbit numbers.

The long-term effect of RHDV

Cooke and Fenner (2002) suggest that the future of RHDV will depend on two characteristics: firstly, on the probability that rabbits develop genetic resistance, and secondly that RHDV retains its high virulence. Furthermore, the authors suggest that retention of high virulence depends on the mode of transmission. Selection for sustained virulence is most probable in scenarios in which airborne vectors contaminated by RHDV are the predominant mode of viral transmission (Cooke and Fenner 2002).

Genetic resistance of rabbits to RHDV has not yet been identified. A recent French study indicated that despite high mortality rates during past epizootics and in particular myxomatosis, no loss of genetic variability in wild rabbits has been observed (Queney *et al.* 2000). RHDV is a relatively young disease in New Zealand and genetic changes in rabbits would not be expected in this short period. A genetically-modified, attenuated strain of *Myxoma virus*, which can express the RHDV major capsid protein and thus protect against both RHD and myxomatosis, was recently developed in Spain (Barcena *et al.* 2000). The recombinant viruses can be safely transmitted by direct contact between rabbits and will be used by conservationists to protect wild rabbits in Europe (Torres *et al.* 2000; 2001). However, the deliberate release of such an agent, as an act of bioterrorism, could have a devastating effect in countries where RHDV plays a useful role in controlling rabbits. Rabbit management has different goals and strategies in different countries and international regulations alone may not fully protect countries from unwanted organisms. Thus it is the scientist who must take responsibility for

reducing the risk of the accidental or illegal spread of any genetically modified rabbit viruses (Angulo and Cooke 2002).

Conclusion

The work undertaken in this thesis illustrates the complexity of RHDV epidemiology in a low density wild rabbit population in New Zealand. The improved understanding of patterns of natural outbreaks in this environment should enhance the application of RHDV as a biocontrol agent in this country. The work is unusual as epidemiological knowledge is typically applied to minimise the impact of animal diseases rather than to promote its spread. The results of this study have indicated areas requiring further research and in particular the importance of carrier rabbits. Successful rabbit control in the future will depend on how knowledge of RHDV epidemiology is integrated into more complex control strategies.

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