Rabbit Haemorrhagic Disease: Wild rabbits show resistance to infection with Czech strain-351 RHDV initially released in Australia.

Final report prepared for Australian Wool Innovation and Meat and Livestock Australia
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7.T.5 RHD: Genetic Resistance.

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Executive Summary: *Challenge tests on seronegative sub-adult rabbits from 9 sites in south-eastern Australia showed that wild rabbits have developed resistance to infection with Czech strain 351 RHDV originally released in Australia. Differences in infection rates were observed between populations and in comparison with domestic rabbits used as an unselected reference population. Selection for resistance to infection appears to be highest in zones of intermediate rainfall rather than arid or high rainfall areas.*

Resistance to RHDV infection may help to explain recent increases in rabbits in inland Victoria and South Australia and has implications for future rabbit control. Nevertheless, there is also a case to argue that RHDV has co-evolved to at least partly off-set the changes in rabbit resistance; wide-spread outbreaks of RHDV are still regularly observed in areas where resistance is high.

In practical terms we have identified those areas where resistance is highest and where more resources will be required in the future for addition rabbit control such as poisoning and warren destruction. Resistance has not reached such high levels that inoculating rabbits with a standard 0.5 ml of stock RHDV suspension no longer causes disease, however this may not be enough to initiate new RHD outbreaks if Czech strain virus is less able to infect resistant rabbits.

Understanding how RHDV coevolves as rabbits develop increasing genetic resistance will be important in assessing the long-term future of RHDV as a biological control agent. Research on the significance of genetic changes in the virus needs to be extended with direct studies on the virulence of viruses currently active in the field.

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Introduction

Wild rabbits, *Oryctolagus cuniculus* (L.) were introduced into Australia in 1859 and within 70 years had become established across the southern two thirds of the continent causing enormous damage to agriculture and the environment. Despite major efforts such as the construction of barrier fences to stop the initial spread, and the development of poisoning and warren destruction to reduce numbers, it was only the introduction of myxoma virus (MV) in 1950 that led to a dramatic and significant reduction of the problem (Fenner and Ratcliffe 1965).

Nevertheless, within a few years it was recognized that rabbits were developing genetic resistance to myxomatosis and that new attenuated strains of virus were evolving (see review in Fenner and Fantini 1999). Mortality of infected rabbits fell from an estimated 99% to about 50% and has since stabilized around that point as the rabbits and virus continue to co-evolve in what appears to be a prolonged 'arms race' with virus virulence adjusting to match gains in genetic resistance.

The decline of myxoma virus as a biological control agent and the consequent partial recovery of rabbit numbers led to the introduction of additional biological control agents, such as the rabbit fleas *Spilopsyllus cuniculi* (Sobey and Conolly, 1971) and *Xenopsylla cunicularis* (Cooke, 1990, Mutze 1996) in an effort to enhance virus spread. Mechanical and chemical rabbit control methods were also re-appraised to increase effectiveness and increasingly applied (Williams *et al* 1995). Although these efforts helped to hold rabbits well below former extremes, it was not until 1995, when Rabbit Haemorrhagic Disease Virus (RHDV) was introduced into the field in Australia that further progress was made in driving the rabbit population down. In arid areas in particular, Rabbit Haemorrhagic Disease (RHD) caused population reductions exceeding 90% (Mutze *et al*, 1998) and

immediate benefits for sheep and cattle production and the environment were observed. Rabbits remained low for the following seven or eight years but since 2003 a steady increase has been noted in carefully monitored populations (P. Sandell, Parks Victoria, unpublished, N. de Preu South Australian NPWS, unpublished, S. McPhee, unpublished). Given previous experience with the development of genetic resistance to MV infection, it was important to know if the resurgence of rabbits was indicative of developing genetic resistance to RHDV.

Recent research in Europe has indicated a possible genetic basis to resistance to RHDV infection and other viruses in the *Caliciviridae*. Ruvoen-Clouet *et al* (2000) provided evidence that RHDV enters cells by initially binding to histo-blood group antigens expressed on the oral and intestinal mucosa of rabbits. It is also known that Noroviruses affecting humans bind to these same histo-blood group antigens and that susceptibility to infection is closely linked to the expression of ABO, Lewis (Le) and secretor (Se) genes. Indeed, resistance against one strain of Norovirus (Thorven *et al* 2005) is conferred by a single amino-acid change on the fucosyltransferase (FUT-2) gene that controls blood-group antigen expression. This has invigorated research on the equivalent gene in European rabbits (P. Esteves, J. LePendu, pers. comm.) because changes in the frequency of its alleles may help track any development of the rabbit's resistance to RHD.

Clearly, in considering the development of genetic resistance to RHDV infection in rabbits, it is important to include the possibility that genetic changes affecting the expression of virus binding sites in mucosal tissues could influence the ability of the virus to enter cells. However, the likelihood that resistance might also involve more general cellular responses should also be considered (Best and Kerr, 2000), especially if these responses prevented severe damage to hepatocytes and resultant disseminated intravascular coagulation which is the main cause of rapid death.

In this paper we describe experiments carried out to see if wild rabbits are developing genetic resistance to infection with Czech strain 351 RHDV originally released in Australia. We also extend our observations to ask whether resistance to infection might involve a simple mechanism such as the restriction of the ability of the virus to bind to surface antigens of the rabbit's mucosa or whether resistance is likely to involve more general cellular immunity.

We are also aware that this initial investigation represents only part of the picture because, as yet, we have no clear idea of possible changes in the relative virulence of viruses circulating in the field. As seems to have been the case for myxomatosis, it will ultimately be the co-evolution of virus virulence and rabbit genetic resistance that determined the long-term effectiveness of RHDV as a biological control agent in Australia.

Methods

General

To detect genetic resistance we followed the strategy used by Marshall and Fenner (1960) of using a standard virus strain to challenge susceptible rabbits from the field and compare their responses with those of unselected laboratory rabbits used as a reference population. In this exploratory study, it was not possible to breed large numbers of rabbits in captivity or consider progressive increases in resistance from year to year. Nevertheless, apart from the direct experimental evidence, we were hopeful of obtaining supporting evidence such as an association between levels of resistance and the frequency and impact of previous exposure to the virus in each sampled rabbit population. Some closely studied rabbit populations were known to have had severe annual outbreaks of RHD in each of the 10 years since RHDV spread while others had been exposed to less severe or fewer outbreaks over the same period.

The analyses of Neave (1999) and Henzell *et al* (2002) are particularly useful in this respect because they showed that the initial impact of RHD across Australia could be partly explained by climatic variables that modified epidemiology. They found that RHD had a greater impact in warm, dry areas than in cool, wet areas and that summer outbreaks of RHD were relatively ineffective in areas where rains fall mainly in summer. There was also evidence of markedly reduced disease impact in some coastal areas on both the eastern and western coasts of the continent.

Subsequent observations show that the initial impact of RHD in any specific area was often indicative of its subsequent performance as a biocontrol agent (e.g. Flinders Ranges (SA) Mutze *et al* 1999, Cooke *et al* 2000; Bathurst (NSW) Saunders *et al* 2000, Moriarty *et al* 2004; Bacchus Marsh (Vic) McPhee, unpublished; Cattai (NSW) Richardson *et al*., in press) although in some areas, following a moderate initial reduction, rabbit numbers have subsequently recovered (e.g. Kojaneerup (WA), Bruce *et al.,* 2004) or declined over many years to become very sparse (e.g. Whetstone (Qld), Storey *et al* 2004).

In general, since the initial spread of RHD, selection pressure for the development of genetic resistance should have been greatest in warm dry winter-rainfall areas where outbreaks occur year after year.

Basic assumptions

In this study it has been assumed that virulent RHD was effectively a new disease in rabbits in Australia despite serological evidence that RHDV-like viruses have been circulating in wild rabbit populations in Australia, New Zealand and Europe for many years previously (Nagesha *et al* 1995, 2000; O'Keefe 1998: Cooke *et al* 2002, Forrester *et al* 2003, Forrester *et al* 2006). A virus closely related to RHDV, called rabbit calicivirus (RCV), is a non-pathogenic enteric virus (Capucci *et al* 1996) and its failure to cause disease has meant that, along with other similar non-pathogenic forms, it has circulated unnoticed until recently.

On the basis that any putative RHDV-like viruses circulating in Australia were considered to be non-pathogenic. We also assumed that wild and domestic rabbits everywhere in Australia were highly susceptible to infection with RHDV at the time it first spread.

Field collection and quarantine

Collection of young susceptible rabbits from the field proved more difficult than was reportedly the case for Marshall and Fenner (1960). For this study it was necessary to consider an additional disease with potential to spread in almost any month, instead of only one disease that occurred fairly predictably in spring. Although we tried to collect rabbits during periods when RHD was normally least active, this was not always possible especially when severe drought across much of south-eastern Australia in 2006 shortened the rabbit's breeding season and made it necessary to collect young rabbits over very few months. Special quarantine procedures had to be established to reduce the risk of inadvertent spread of myxomatosis or RHD between newly captured rabbits and also during transport to the laboratory and subsequent maintenance until they reached the minimum age for testing (12 weeks).

Basically, rabbits were caught in carrot or oat-baited wire cage traps set on active rabbit warrens and, if they were about 4 – 8 weeks old with a low probability of prior infection with RHDV, they were placed in plastic boxes (400 mm x 300mm x 300 mm high) with an insect-proof mesh top. The rabbits were kept isolated with the exception that, where very small rabbits from the same litter were caught in the same trap, these were subsequently kept together. Below a wire-mesh floor within the box a bed of wood shavings or pet litter was provided to absorb urine. Rabbits were inspected at least daily when they were fed and more closely examined when boxes were cleaned every few days. As wild rabbits normally do not drink, newly captured rabbits were always provided with an excess of fresh carrot to ensure that they did not become dehydrated while adjusting to a diet of dry food pellets and water.

A 1 ml blood sample was collected from an ear-vein of each rabbit and rabbits were weighed, sexed and marked with individually numbered ear-tags. Rabbits were also treated with flea-powder to eliminate fleas that could transmit viruses. To maintain quarantine, each rabbit was placed in a clean calico bag for weighing and blood sampling and non-disposable equipment was sterilized by washing with disinfectant between uses. Personnel wore disposable gloves and coats or aprons and showered and changed clothing before feeding rabbits if they had previously been handling rabbits or blood samples. Rabbits that became ill after capture were euthanized and, as for any that died suddenly, autopsied with liver and other tissue samples being taken as necessary to confirm the cause of illness (most frequently RHD or myxomatosis). Body weight was used to estimate the approximate age of each rabbit on the basis that they grow at about 10 g/day after leaving the nest. On coming above ground 20 days after birth, young rabbits weigh about 200g; a 400 g rabbit is considered to be about 40 days old.

Sera extracted from blood samples were frozen in individually labelled microcentrifuge tubes and sent overnight packed in ice to the Animal and Plant Control Group laboratory (APCL) in Adelaide for RHD ELISA testing (see *Antibody tests* below). Results on the serological status of each rabbit were provided by fax or email within 48 hours. The rapidity of testing helped to reduce the amount of time that rabbits needed to be kept under field conditions at remote locations. Only rabbits suitable for testing were retained; the remaining rabbits were euthanized.

Susceptible rabbits, still in individual boxes, were then taken to the Queensland Department of Primary Industries and Fisheries, Robert Wicks Pest Animal Research Centre, Inglewood by car, air-conditioned as necessary to avoid heat stress. Fresh carrot was provided as a water source while transporting rabbits to avoid spills and wet cage floors. On arrival rabbits were transferred to standard laboratory cages and held singly or in small groups. Rabbits from different sites were kept in separate banks of cages to minimize risk of disease spread, especially when newly introduced into the laboratory. Constant vigilance was needed because signs of myxomatosis did not necessarily become apparent until 8 – 9 days after initial infection and there was always a risk of spread of sub-clinical RHD or transmission of non-pathogenic RHDV-like virus.

Some inadvertent spread of myxomatosis and apparently, RHDV-like virus, did occur during transport or when rabbits were first introduced into the laboratory. This was revealed when rabbits were routinely monitored and blood sampled to check their health or during pre-trial blood sampling. Rabbits that showed positive titres in RHDV ELISAs were excluded from trials and this reduced the size of some samples below the minimum of twelve rabbits sought.

Young rabbits frequently have circulating antibodies of maternal origin in their sera and, as these would have interfered with experimental challenge, rabbits were not tested until they had reached 12 weeks of age and had lost all traces of maternal antibody protection (Cooke *et al* 2000, Robinson *et al* 2002).

Sites where rabbits were obtained for testing

Rabbits were obtained from the following sites, listed in order of collection. Annual average rainfall was assigned for each site using the BIOCLIM data base (Nix 1986):

Yanyanna Flinders Ranges National Park (SA). 31º 27’ 17” S, 138º’ 38” 10 E, Open rangeland on calcareous loam, 315 mm annual average rainfall, RHD antibodies in one rabbit, regular annual outbreaks of RHD. Rabbit population increasing.

Turretfield (SA). 34º 33’ 00” S, 138º 49’ 47” E, Farmland, cereal and sheep production red-brown earth. 350 mm annual average rainfall. No evidence of RHD when trapping rabbits although outbreaks occur roughly every second year. Rabbit population stable.

Hattah, Hattah-Kulkyne National Park (Vic). 34^0 38' 03" S, 142^0 24' 60" E, Pine-Buloke woodland on red sand dunes, 304 mm annual average rainfall, several cases of RHD among trapped rabbits. RHD occurs each year. Rabbit population increasing.

Ingliston, near Bacchus Marsh (Vic). 37^0 39' 03" S, 144º 19' 24" E. Sheep and wool production Open pasture on gravelly soils around granite outcrops. 540 mm annual average rainfall. RHD outbreak less intense than at Hattah or Spring Hills. RHD occurs each year. Rabbits very abundant and increasing.

Yambuk (Vic). 38º 19' 51" S, 142º 02' 38" E, Coastal dune and underlying limestone with mixed introduced pasture and remnant coastal shrub vegetation alongside wetlands. 754 mm annual average rainfall. RHD antibodies in 7 of 33 captured rabbits. Rabbit population of moderate density but stable.

Spring Hills, near Bendigo (Vic), 37º 01' 03" S, 144º 22' 25" E, Sheep and wool producing area. Open pasture on gravelly soils around granite outcrops. Numerous cases of RHD among trapped rabbits, annual average rainfall 673 mm. Rabbits common around granite outcrops.

Michelago (NSW). 35º 44' 43" S, 149º 08' 59" E. Sheep and wool production Open pasture on granitic sands. Annual average rainfall 657 mm. RHD antibodies in a few rabbits, no deaths among captured rabbits. Rabbits abundant in localized areas.

Valpine, near Bathurst (NSW) 33º 21' 36" S, 149º 22' 48" E. Sheep and wool production. Open pasture with scattered Eucalypts on sandy granite soil. Annual average rainfall 701 mm. RHD antibodies in a few rabbits, no deaths among captured rabbits. Rabbit population locally abundant but stable.

Bulloo Downs (Qld). 28º 36' 06" S, 142º 39' 37" E. Red sand dunes alongside water courses intermittently flooded by Bulloo River. Annual average rainfall 195 mm. Main land use, cattle production. No evidence of RHD in collected sample of rabbits obtained from a small nucleus of rabbits that had persisted after a major warren ripping campaign in 2001.

Locations of these sites are shown in figure 1. Attempts to trap rabbits at Whetstone, an RHD study site in Queensland, were unsuccessful during this project because rabbits had become extremely rare since RHD spread (Story *et al* 2004). However, rabbits have recently been obtained from nearby sites and are being assessed for future challenge tests.

General laboratory maintenance of rabbits

In the laboratory, rabbits were kept in banks of large metal cages (480mm x 660mm x 480 mm high) with open wire front and provided with commercial rabbit grower pellets and water *ad lib*. Carrots were provided every few days. A smaller metal nest box in each cage enabled wild rabbits to hide. Staff maintaining the rabbits wore laboratory coats,

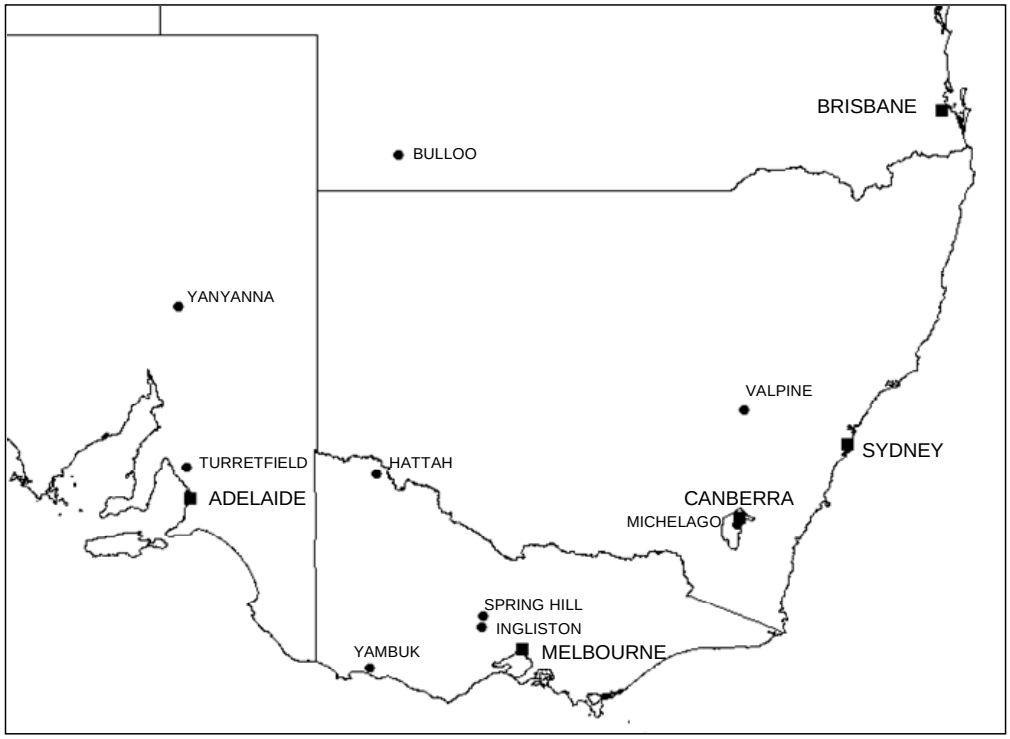

Figure 1. Sites where rabbits were trapped for challenge testing (●).

rubber gloves and boots, stepping through disinfectant foot baths on entering each room housing rabbits.

When rabbits were old enough to be transferred into experiments, the cages were thoroughly washed and disinfected before accommodating incoming rabbits.

Domestic (control) rabbits

Domestic rabbits used as experimental controls were obtained from Mr Ian Handebo, Armidale, NSW. Purchased at 12 weeks of age, these rabbits had lost any maternal antibodies they might have acquired from their immunized dams and invariably proved seronegative on routine RHD ELISA tests. The original stud rabbits had been bought from Growtec Pty Ltd in 2005 and enquiries (Daniel Brown, Growtec Pty Ltd, pers. comm.) confirmed that there had been minimal exposure of the original colony to RHD although two small outbreaks had been detected and stamped-out.

Virus

To standardize tests we used newly opened vials of Czech strain-351 RHDV (CSIRO Wildlife and Ecology Batch RCV-1B), commercially available in Australia. This stock virus, containing 3000 LD_{50} rabbit doses/ml, was maintained deep-frozen until shortly before use. After thawing and extensive shaking to ensure the suspension was well mixed, it was diluted as necessary in sterile phosphate buffered saline (PBS) and administered in a standard dose of 0.5 ml.

Antibody tests

In the APC laboratory serum samples were analysed using a series of ELISA tests (competition ELISA, IgG, IgM and IgA) that allowed classification of rabbits as: seronegative to RHD, seropositive with antibodies of maternal origin, seropositive with antibodies to a related RHDV-like virus or seropositive survivors of RHD (Capucci *et al* 1991, Cooke *et al* 2002).

Determination of oral challenge dose

Prior to commencing the trials, domestic rabbits were given several days to adjust to living in individual plastic boxes (470 mm x 330mm x 270 mm high) with insect-proof wire tops. Each box was fitted with a water bottle and container for food pellets and a pet-litter bed below a mesh floor to absorb urine and spilt drinking water. The room in which the rabbits were held was maintained at 22 ± 2ºC on a 12 hour light/dark cycle.

Blood samples had previously been collected from each rabbit and frozen sera sent by overnight courier to the APC laboratory for ELISA tests (see *Antibody tests* above) to confirm that all were seronegative.

A series of dilutions (e.g. 1: 10, 1:20, etc) of the commercial RHDV suspension in PBS was prepared and 0.5 ml of each dilution was orally administered to 5 domestic rabbits. A 1-ml tuberculin syringe without needle was used for dosing, the syringe being introduced through the diastemma to place the dose on the tongue.

Once inoculated with RHDV, rabbits were inspected every 8 hours (routinely 7 am, 3 pm and 11 pm) to record signs of infection and time of death. Rabbits that died were removed from test boxes at the time of routine inspections and rectal temperature taken to obtain an approximate time of death based on the recorded rate of cooling of cadavers. They were then autopsied and signs of RHDV infection were noted (e.g. pale liver with reticulate pattern, swollen spleen and haemorrhages in the lungs) and a liver sample and blood sample collected for ELISA tests.

When inspecting and feeding rabbits or changing rabbits from dirty boxes to clean ones, personnel changed gloves and washed hands between rabbits to minimize the risk of cross infection. Staff wore laboratory coats and rubber boots and stepped through disinfectant foot baths on entering the rooms where viruses were being used. Personnel involved in experiments involving live virus were not involved with daily maintenance and feeding of other rabbits and indeed did not enter rooms where other rabbits were held, further reducing risk of infection.

At the end of the trial, 2 weeks after inoculation, surviving rabbits were killed using Lethobarb (60 mg/kg) after tranquilization (Zoletil 15 mg/kg and Xylazine 5 mg/kg) and a final blood sample taken by heart puncture at the same time that samples of blood and liver were collected, labelled and prepared for storage. Dead rabbits were sealed in plastic (biohazard) bags before removal from the laboratory and incineration.

Rabbits that became infected during these trials were defined as those that seroconverted but survived as well as rabbits that developed acute disease and died or were euthanized. Data were analysed in a generalized linear model assuming that the probability of infection would potentially be explained by virus dose rate, sex and body weight as found in earlier studies e.g. Cooke and Berman (2000). On the basis of the results obtained we selected a dilution that would infect a major proportion of the control rabbits yet not be so excessive that it might mask limited genetic resistance in the wild rabbits.

Experiments to detect evidence of resistance

Groups of wild rabbits 12 weeks old or more and seronegative on ELISA testing were challenged by dosing them orally with 0.5 ml of a 1:25 dilution of stock RHDV suspension. The procedure used was identical to that described in *Determination of oral challenge dose* above except that an eyeball from each rabbit was also collected and preserved in 10% buffered formalin as a means of checking the age of rabbits based on dried eye lens weight following the method of Dudzinski and Mykytowycz (1961). Domestic rabbits as experimental controls were dosed at the same time although smaller numbers were used on the basis that a large data set for comparison would accumulate with repeated tests.

Mortality, infection rates and survival times of wild rabbits were compared as appropriate using χ^2–tests (g-test) and Student *t*-tests after correction against the critical explanatory variables as determined from the trials with unselected laboratory rabbits.

Preliminary observations on the mechanism of resistance

Some wild rabbits that survived challenge with an oral dose of virus remained seronegative with no evidence of infection with RHDV. These might have simply avoided infection because of changes in virus binding sites on the mucosal surface and a simple way to test this was to challenge such rabbits with an equivalent dose of intramuscularly administered virus, effectively by-passing the mucosal barrier.

Rabbits chosen for such trials were inoculated in the large muscle mass on the hind leg with 0.5 ml of a 1:25 dilution of RHDV and monitored over the following 14 days as described for the main challenge tests. Infection was indicated by the development of acute RHD or seroconversion within 2 weeks. Remaining rabbits were euthanized at the end of this second experimental period.

Comparative data related to earlier published work

The use of domestic rabbits as an unselected reference population was not ideal, given their larger body size and genetic background, but unfortunately there was no alternative such as a laboratory colony of unselected wild rabbits. Nevertheless, Cooke and Berman (2000) had previously challenged laboratory-bred rabbits derived from stock collected in the Canberra region before RHD spread, and it was considered that more recently collected data could potentially show changes against the baseline information.

On that basis, a group of 11 seronegative rabbits from Michelago (near Canberra) were challenged orally with the same virus dose (0.5 ml, undiluted RHDV preparation or 1500 LD_{50}) as Cooke and Berman (2000) had used. As with other experiments, rabbits were closely monitored to measure survival times, infection and mortality.

Mortality, infection rates and survival times were compared using χ^2–tests and Student *t*-tests as appropriate.

Results

Need for a new test protocol

When the testing was first envisaged we considered that it may be possible to test rabbits using methods previously used to evaluate RHD (e.g. Cooke and Berman 2000) to see whether rabbits had developed overt resistance to challenge. We could then use published data as a base-line for comparisons. In line with the observations of Marshall and Fenner (1960) we considered that resistance might be indicated by lower case mortality rates and extended survival times among the rabbits.

Twelve rabbits from Yanyanna were challenged intra-muscularly with 1500 LD_{50} Czech strain 351 virus and all experimental rabbits developed acute RHD. All rabbits died within 36 – 134 hr of inoculation (mean 60.4 hr) and all showed at least one of the typical signs of RHD; haemorrhages and congestion of the lungs, pale liver with a reticulate pattern or a swollen and enlarged spleen. ELISA tests on blood samples confirmed the presence of virus in each rabbit.

After correction for sex and body weight effects, survival times were equivalent to those obtained previously by Cooke and Berman (2000) for intra-muscularly inoculated wild rabbits (mean survival time 52.7 hr, range 29 – 114 hr).

With this clear indication that overt resistance to a high intramuscular dose of RHDV had not developed, emphasis changed to ask whether wild rabbits nonetheless showed evidence of resistance at lower doses of virus. This involved development of a new test protocol as follows.

Derivation of oral challenge dose

Using the methods described above (*Determination of oral challenge dose*) we analysed data from 45 domestic rabbits (including 5 zero dose controls). It was found that both virus dose (expressed as the equivalent i.m. LD_{50} at each dilution) and sex of rabbits were the only variables among those measured that significantly influenced the probability of infection. Body weight was not significant, thus removing one of the perceived problems in extrapolating results from larger domestic rabbits to smaller wild ones.

The final fitted regression was:

Infection probability (p) = 0.1709 (± 0.1710) + 0.2525 (± 0.1096)*$\log_{10}$ (dose+1) - 0.2998 (±0.1390)*(sex)

where female = 0 and male = 1.

Despite dose and sex being statistically significant explanatory variables, the regression (F = 6.28, p = 0.004) only explained about 40% of the variance of the data, presumably because of large variation in the responses of individual rabbits.

Twenty-one of the twenty three domestic rabbits that became infected in these trials died of RHD. This mortality rate (over 90%) is similar to that observed as RHD first spread among susceptible rabbits (see Cooke and Berman (2000) for review).

For experimental purposes we selected a 0.5 ml oral dose of a 1:25 dilution of the stock virus suspension (equal to i.m. 60 LD_{50}) adequate to infect about two thirds of unselected domestic rabbits.

Comparative tests using wild rabbits

Comparative tests using the selected oral dose (0.5 ml, 1:25 dilution of stock RHDV preparation) were carried out using wild rabbits from 9 sites. The results are summarized (Table 1) and show the proportion of rabbits from each population that became infected (i.e. seroconverted or developed acute RHD). Although sex of rabbits was an important variable influencing the infection rate in domestic rabbits, we found no evidence of differences in infection rates between sexes in wild rabbits. Overall, 15/43 female rabbits and 19/52 male rabbits became infected during challenge trials ($\chi^2 = 0.3$, n.s.).

As a consequence, rather than adjusting for the numbers of male and female rabbits in each sample to calculate the infection rate that might have been expected we have used an average value. The difference between this estimated value and the observed proportion of rabbits infected provides the best indication of change attributable to selection for resistance to infection with Czech strain 351 RHDV.

Table 1. Infection rates among wild rabbits in comparison to the rates expected for equivalent samples of unselected rabbits. Annual rainfall is included as a single variable to distinguish climatically different sites; it is directly related to rabbit productivity and inversely linked to RHD mortality (i.e. mortality declines in wetter areas).

Site	Rabbits challenged	Infected	Infection rate observed	Infection rate expected*	(E-O) Difference	Annual Rainfall
Bulloo	11	8	0.727	0.649	-0.078	195
Hattah	7	1	0.143	0.649	0.506	304
Ingliston	7	0	0.000	0.649	0.649	540
Michelago	12	3	0.250	0.649	0.399	657
Spring Hills	11	5	0.455	0.649	0.194	673
Turretfield	14	3	0.214	0.649	0.435	350
Valpine	12	7	0.583	0.649	0.066	701
Yambuk	11	7	0.636	0.649	0.013	754
Yanyanna	11	2	0.182	0.649	0.467	315

* based on domestic controls

The differences between estimated and observed levels of infection show no direct correlation with rainfall and so the idea that the selection for resistance might simply reflect the initial disease impact is not well supported. However, if more complex models are explored, it is arguable that the rate of selection for resistance has been low in arid areas, occurred most rapidly in intermediate rainfall zones and declined again in high rainfall areas (Figure 1). The fitted polynomial ($y = ax^2 + bx + c$) explains over 80% of the variance but is not described further given the few data points available.

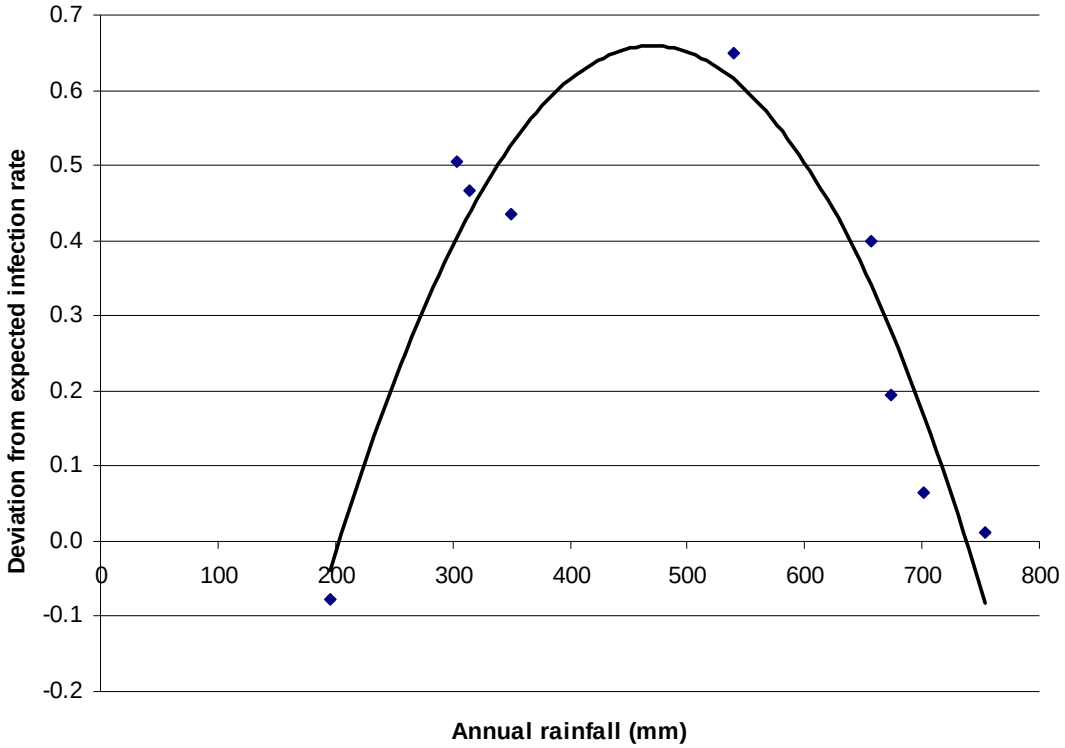

Figure 2. Fitted curve exploring the data, suggesting that selection for resistance has occurred most rapidly in intermediate rainfall zone.

In these experiments 34 of 36 wild rabbits that became infected with RHDV died (94%). So, despite apparently reduced chances of infection, RHD still remains a highly lethal disease once rabbits become infected.

Preliminary considerations of the mechanism of resistance

Five seronegative rabbits from Turretfield were dosed orally with 0.5 ml undiluted virus preparation (equivalent to 1500 LD_{50}) and only 2 died. Although the sample size is small, this mortality is significantly less ($\chi^2 = 7.47$, $p < 0.01$) than expected from previous experiments with orally dosed unselected laboratory-bred wild rabbits where 12/12 rabbits contracted RHD (Cooke and Berman 2000). However, when the 3 survivors, still seronegative, were again inoculated with the same dose of virus intramuscularly, a further two died from RHD. The remaining rabbit survived but again showed no evidence of sub-clinical infection such a seroconversion. The rabbits that died after intramuscular inoculation had clearly resisted infection by the oral route and had not sustained an infection or developed protective antibodies or cellular immunity on their first exposure to the virus.

Wild rabbits that survived oral challenge with a low dose of virus (1:25 dilution RHDV suspension) and showed no evidence of seroconversion were re-challenged with the same dose given intramuscularly and a significant proportion of these rabbits contracted

RHDV. Although care must be taken in using small samples, in general, the probability of rabbits from any given site becoming infected following intramuscular challenge is correlated ($F = 8.403$, $p < 0.01$) with the probability of succumbing to previous oral challenge. This might be expected as intramuscular challenge effectively delivers a higher dose of virus, but the observations also imply that evolving mechanisms to resist infection at the mucosal barrier are paralleled by, or part of, more general resistance. The data shown in Figure 2.

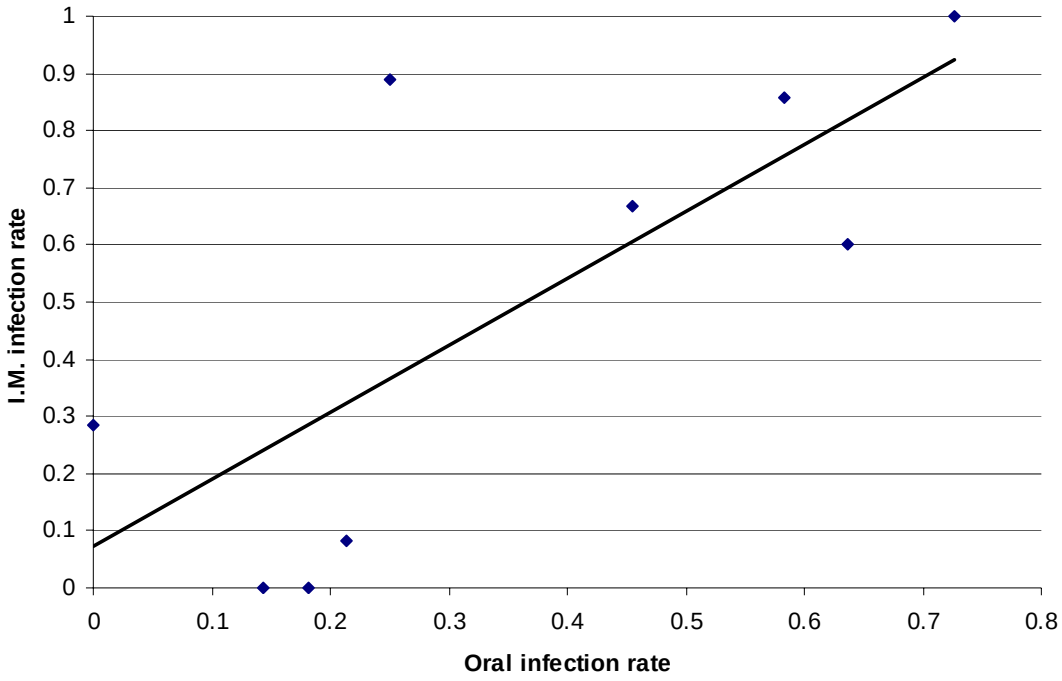

Figure 2. Relationship between oral infection rate of main sample from each test site and the intramuscular infection rate of those rabbits that did not become infected following initial oral challenge

Comparative data related to earlier published work

All eleven rabbits collected from Michelago near Canberra died following oral inoculation with 0.5 ml stock RHDV suspension. Survival times averaged 64.4 ± 3.9 hr and, after downward correction for body weight effects, this result is lower than the 72.5 ± 9.4 hr obtained for laboratory-bred wild rabbits originating from parents collected before the spread of RHD through the Canberra area (Cooke and Berman 2000).

Discussion

We established a broad relationship between the quantity of virus administered orally and the infection rate in domestic rabbits. With low but increasing experimental dose, the proportion of seronegative domestic rabbits that contract the disease rises relatively sharply at first and then more slowly.

A dose-dependent response to viral challenge requires further thought. It might be expected, for example, that even a few viral particles reaching binding sites on the gut or respiratory mucosa could initiate infection. However, the histo-blood group antigens to which RHDV binds (Ruvoen-Clouet *et al*, 2000) not only occur on the surface of mucosal cells but they may also be secreted in body fluids such as saliva and milk. Ruvoen-Clouet *et al* (2006) have shown that, in human milk, these secreted histo-blood group antigens can effectively bind with Norovirus particles and reduce infection risk. Presumably, factors such as the suitability of binding sites and secretor status of different rabbits provides much of the variation that leads to differences in resilience to infection even among unselected rabbits. Nevertheless, with increasing virus load it seems likely that some viral particles would eventually reach the binding sites on mucosal cells and initiate a general infection. Even so, it would be unwise to assume that all resistance is associated with factors operating at the mucosal surface.

The results using domestic rabbits imply that even in populations with little, if any, previous exposure to RHDV there is considerable variation in response to challenge with the virus. In the context of the introduction of highly pathogenic RHDV into Australia, it might be expected that rabbit populations would be under considerable selective pressure and that genes conferring protection from severe infection would quickly be selected, reducing the impact of RHD.

Indeed, challenge trials using wild rabbits showed that rates of infection following oral challenge with low doses of RHD were often substantially lower than those seen in domestic controls. However, the results were not uniform. In some populations all rabbits tested resisted infection with Czech strain-351 RHDV but in others a high proportion still became infected inferring that there had been little selection.

Nevertheless, despite these seemingly straight forward results, the data raise a further problem. At this stage we have demonstrated increased resistance to infection, not increased ability to recover from infection. Indeed, there was no evidence that wild rabbits have prolonged survival times after infection as might be expected if they had been selected to better withstand infection. As a result it is not clear how resistance to infection confers the reproductive advantage that would drive its selection unless some rabbits simply do not become infected at doses normally encountered in the field. That is, in resistant rabbits the dose response curve may not only have shifted to the right of the curve for unselected controls but it may also have a lower slope or lower asymptote that would mean that many rabbits would simply not contract the disease.

The work of Hall *et al* (2005), although drawn from the field of radiation science, provides some insight for both understanding the dose-response curve in mammals and for developing a picture of possible dose response curves in selected populations. Hall *et al* (2005) discuss how genotype affects the response of genetically susceptible mice and normal mice exposed to radiation. Ninety percent of a line of genetically susceptible mice developed cataracts after a low dose of radiation whereas less than 10% of wild-types showed ill effects within the duration of the experiment. In other words, their data

support the suggestion above, that the curve showing response to a given challenge in resistant rabbits would not only be pushed to the right but could have a lower asymptote. If this was the case, and more highly resistant rabbits entered the breeding population, we would predict an increase in the proportion of adult rabbits in the population that were seronegative to RHDV. Discerning such animals might be difficult if serological data was clouded by (a) antibodies to RHDV-like viruses and (b) genetic changes in field strains of RHDV that enable them to adapt to match changes in the host resistance. Nonetheless, it is clearly important to look for such changes in field data to see if there is evidence that some adult rabbits in the breeding population remain seronegative to RHDV.

It might be expected that wild rabbits from warm, dry winter-rainfall areas should show lower rates of infection than rabbits from cooler wetter areas and summer rainfall areas if selection for resistance to infection was related to the past intensity of disease outbreaks (Henzell *et al* 2002). However, there are equally compelling conceptual models (Figure 2) suggesting that the rate of selection for resistance to RHD may not only be influenced by disease impact, which declines in wetter areas, but may also be influenced by other factors that reduce the rate of selection among rabbits in arid areas. Natural selection, for example, might occur most rapidly where conditions permit not only high RHD mortality but also enable prolonged breeding of rabbits with resultant high productivity and high population turn-over. In the Flinders Ranges, near Yanyanna in South Australia, for example, productivity was low and the impact of RHD was so high that there was very low recruitment of young rabbits into the breeding population. For 3 – 4 years after the initial RHD out-break in late 1995, the small breeding population was dominated by rabbits from the 1995 cohort that had simply been lucky enough to have survived RHD because they were young when first infected (Mutze *et al* 1998, Cooke unpublished). These immune rabbits survived well and any selection for resistance would have been substantially diluted while they persisted. By contrast, in the highly productive population at Ingliston, Victoria, RHD caused lesser mortality but few adults survived into a second breeding season and this should have facilitated faster genetic change in the population. Current analyses of field epidemiological studies on these two populations may provide additional insight into these processes (Butler, Yoon and McPhee, unpublished).

Although we assume that all Australian rabbit populations (including domestics) were equally susceptible when RHD first spread, it is arguable that some wild rabbit populations might have naturally differed in their resistance to infection even before RHD was introduced. However, data from Asgari *et al* (1998) suggest that wild rabbits from the Adelaide region were initially much more susceptible to Czech strain 351 RHDV infection than is currently the case. In their experiments it was shown that a single fly-spot containing virus equivalent to 2 -3 i.m. LD_{50} (equivalent to 0.5 ml of a 1:500 dilution of stock solution) was sufficient to cause infection in two orally-dosed wild rabbits that were captured near Adelaide. One of these rabbits survived infection and seroconverted but the other died. The virus in five fly-spots, equivalent to 10 - 15 i.m. LD_{50}, (1:100 dilution of stock solution) was enough to infect and kill both wild rabbits in the same trial and doses made with higher numbers of fly spots also killed all wild rabbits challenged. However, the present test dose used in our experiments, a 1:25 dilution of

stock solution, is equivalent to 60 i.m. LD_{50} and yet infected only 3/14 seronegative rabbits captured at Turretfield (near Adelaide) in 2006.

This supports the argument that rabbits in the Adelaide region are now more resistant to infection with Czech strain 351 RHDV than was previously the case. It is also notable that when five seronegative rabbits captured at Turretfield in 2006 were orally challenged with a standard 0.5 ml dose of RHDV (1500 LD_{50}) only two became infected. This suggests that the Turretfield rabbits may be becoming overtly resistant to oral infection at least, although subsequent inoculation of 1500 LD_{50} by the intra-muscular route caused infection in two of the three seronegative survivors from the first trial. The surviving rabbit showed no evidence of infection and did not develop antibodies to RHDV despite exposure to large amounts of the virus by two inoculation routes.

Nevertheless, similar challenge experiments on seronegative rabbits from Michelago south of Canberra resulted in acute infection of all 11 rabbits following a 0.5 ml oral dose of undiluted virus preparation (1500 LD_{50}). If anything, survival times were significantly lower than those obtained by Cooke and Berman (2000) using rabbits bred in the laboratory from breeding stock captured before RHD first spread. This reinforces the idea that the direction of natural selection is towards avoidance of infection rather than greater tolerance of the virus and the development of mechanisms to stop the virus causing a generalized disease. Rabbits in the Canberra area have not developed sufficient genetic resistance to withstand a severe RHDV challenge and seemingly, development of genetic resistance to RHDV infection is not as advanced at Michelago as it is in the Adelaide region.

Rabbits from Bulloo, Valpine and Yambuk do not appear to differ from unselected domestic controls in terms of resistance to infection with Czech strain-351 RHDV. This might imply that there has been no selection for resistance in rabbits at those sites, although it could also be argued that domestic rabbits are not ideal controls in this instance. A final conclusion remains unattainable without a reference population of wild rabbits against which the development of genetic resistance can be measured. Possibly wild rabbits from isolated populations that still remain free of RHD, such as those on the Kerguelen Islands (Cooke *et al* 2004) or Macquarie Island (Mutze unpublished), might be used.

The fact that many rabbits that survived oral challenge subsequently succumbed to intramuscular inoculation with an equal dose of virus, confirms that they had not become infected on previous oral exposure and developed protective antibodies or cellular immunity. However, this falls short of clearly demonstrating that the resistance of wild rabbits has increased because of selection of genes that prevent virus binding on the mucosal surface of the rabbit's gut and respiratory tract. It certainly remains possible that genetic changes might have reduced the capacity of the virus to bind to those mucosal cells in some rabbits however we must also acknowledge that, in by-passing the mucosal barrier with intramuscular inoculation, the effective dose of virus was also increased. As yet we do not have adequate data to distinguish between these possibilities. However, the correlation between oral infection rate and the intra-muscular infection rate of rabbits that

survived oral challenge suggests that development of resistance at the mucosal surface and a more general cellular resistance are likely to have developed in parallel or be part of the same process. It is also clear that occasional rabbits withstood very high doses of virus by both oral and intra-muscular routes of inoculation implying that they had a generalized cellular resistance to infection and were not reliant on the mucosal barrier alone.

There is now a strong case to argue that wild rabbits from some sites in south-eastern Australia show significant innate or genetic resistance to infection when relatively low doses of Czech strain 351 RHDV are given orally. Further, there is evidence that this resistance has developed over the last decade in response to high selective pressures following the release of RHDV as a new biological control agent. The results indicating the development of genetic resistance to RHD are not surprising seeing that Australian wild rabbits developed significant genetic resistance to myxoma virus infection within a decade of its release (Fenner and Fantini 1999). During early observations on the development of resistance to MV it was also noted that the evolution of resistance had been more rapid in north-western Victoria than elsewhere, possibly because climatic factors influence disease development and survival (Marshall 1959, Williams *et al* 1990). Rendel (1971) provides a broad theoretical framework covering some of the variables that might influence the rate of selection of resistance but did not address that issue specifically.

It is premature to try to predict the consequences of the observed build-up in resistance. There are three possible courses that evolution of the disease could take. First, it could be argued that rabbits will eventually become completely resistant to RHDV infection and the disease will slowly become more and more restricted to limited areas of the continent before becoming ineffective. RHDV is a small RNA virus and so may lack the genetic repertoire enabling it to counter gains in rabbit resistance with adaptive changes in virulence (LeGall-Recule *et al* (2003) have stated that RHDV may have limited capacity to evolve quickly because of its small size and consequent close inter-relation between structural and functional components. This combination of factors would mean that rabbit resistance might out-strip virus virulence so reducing the effectiveness of RHDV as a biological control agent.

A second possibility is that selection for resistance in rabbits will eventually contain RHD as a generalized disease, as appears to be the direction of co-evolution for wild rabbits and MV (Best and Kerr 2000), and it will increasingly become restricted to tissues of the gut, similar to RCV which is a non-pathogenic enteric virus.

Third, the selective advantage that RHDV appears to have over other non-pathogenic RHDV-like viruses is that huge numbers of viral particles are produced following infection and this usually results in the death of the host. Both factors enhance the chances of infecting another rabbit as infection is possible by oral, sub-cutaneous or intramuscular routes and rabbit to rabbit contact and the excretions of carrion eating flies have been indicated as important (Asagari *et al*. 1998). RHDV might retain this capacity

if it can co-evolve by becoming more virulent or evading host defences as host resistance to infection develops.

Information from current studies supports the first or third possibilities in the sense that the lowering of the infection rate is not associated with an increase in sub-clinical infections or extended survival times in infected rabbits. We recorded 2 sub-clinical infections in 23 infected domestic rabbits and only 2 sub-clinical infections among 36 wild rabbits experimentally infected (not significantly different) despite the fact that wild rabbits now show evidence of resistance in some populations.

It is important to remember that results so far relate only to the strain of the virus originally released in Australia (Czech strain-351). In the field there has almost certainly been selection of new RHDV strains that remain capable of lethally infecting rabbits that have developed a degree of resistance to the original virus. Indeed, when we were collecting young rabbits for testing, a widespread outbreak of RHD was observed. Fresh carcases of rabbits that had been killed by the disease were observed over some weeks at many sites and the known epizootic eventually extended from Ingliston and Spring Hills in central Victoria to Yanyanna in the Flinders Ranges of South Australia, over an area of perhaps 100,000 km^2. An outbreak of this magnitude would seem unlikely if resistance was severely reducing the capacity of the virus to infect rabbits.

It may be possible to distinguish between the first and third possibilities listed above by looking at the serological status of adult rabbits in the field. On the one hand, if the proportion of adult rabbits with antibodies to RHD is declining then it may be possible to say that rabbits are slowly out-stripping the virus in terms of resistance to infection. On the other hand, if over 95% of adult rabbits continue to show antibodies to RHD then it will be more likely that the virus is co-evolving to off-set increasing resistance to infection in the rabbits. Nevertheless, there are also other factors that could lead to a reduction in the proportion of adult rabbits showing antibodies such as the development of new strains of the virus.

Small changes in the genome of RHDV samples in Europe have been recorded with some genetic clusters of virus replacing others over time (Nowotny *et al* 1997, Le Gall *et al* 1998) raising the possibility that virus strains could be evolving in response to changes in resistance to infection in the rabbits. However, the situation in Europe is complex both because viruses closely related to RHDV have apparently circulated there since antiquity (Forrester *et al*., 2006) and because the large domestic rabbit industry and associated vaccination programs may be a more important force in RHDV evolution than naturally spreading viruses in wild rabbit populations. Schirrmeier *et al* (1999), for example, detected new virus variants when investigating RHDV vaccination failure in domestic rabbits in Germany and Capucci et al (1998) described the first virus sub-type (RHDVa) that subsequently replaced other strains of RHDV across Europe.

Genetic data on RHDV are being collected in Australia (Sinclair, Mutze, Kovaliski and Esteves, unpublished). As in New Zealand, new variants show relatively few sequence changes from the original Czech strain 351 that was released (Forrester *et al* 2003) but

nonetheless it is possible that significant changes in virulence may also result from very small genetic differences (Capucci *et al* 1998). The situation in Australia and New Zealand, where a known virus strain was deliberately released and there is a relatively small commercial rabbit industry, potentially simplifies studies to understand the co-evolution of rabbits and RHDV.

Recent work on the development of infectious cDNA clones of RHDV capable of infecting cell-cultured rabbit kidney cells (Liu *et al* 2006) may provide a means of exploring the significance of changes in the RHDV genome to determine the direction of changes in virus virulence. However, results from this current study on testing genetic resistance also open up possibilities that might be investigated using careful field observations. For example, it would be interesting to see whether changes in the genetic sequences of RHDV have been more extensive in those areas where rabbits show the highest levels of resistance to infection with Czech strain 351 RHDV.

In the short-term however, the methodology developed in the current studies could be adapted to assess the virulence of recently collected field isolates of RHDV, comparing their infectivity at low dose with that of Czech strain-351 in unselected domestic rabbits and wild rabbits from a site where resistance to infection is high.

Practical implications

While it is important to understand how genetic resistance to RHD in wild rabbits might be developing and consider the long-term implications, there are a number of immediately practical implications from this work. These include both research priorities and implications for action required for dealing with the agricultural and ecological consequences of a resurgent rabbit population.

Research

1. Natural selection appears to be favouring rabbits that avoid infection rather than enabling more to recover from infection. Evidence of steady increases in rabbit populations at sites like Hattah, Yanyanna and Ingliston support the idea that this may be linked to reproductive advantage. Nevertheless, it must be confirmed that resistance to infection actually enables higher recruitment of rabbits into the breeding population.

2. It is important to determine whether changes in rabbit resistance to infection will eventually outstrip virus virulence or whether the assumed ‘arms race’ between virus virulence and rabbit resistance will maintain RHD as a lesser but nonetheless important agent for rabbit control into the future.

3. Data are limited with rabbits from only nine sites so far tested. It will be useful to test batches of seronegative rabbits from additional sites to build up a more comprehensive picture. The use of domestic rabbits as controls has been adequate for our work so far but we have no verified base-line information on the levels of

resistance in wild rabbits from the time when RHDV was first introduced. Data from a wild population not previously exposed to RHDV would be useful.

4. Challenge tests should be repeated every few years using seronegative rabbits from some of the populations included in the present study. This would enable measurement of the rate of evolution of genetic resistance in rabbits and up-grading of advice on rabbit management.

5. A suitable theoretical framework should be developed to extend these preliminary studies on rabbit resistance and rabbit-virus co-evolution. In particular, information on genetic studies related to virus binding sites should be included and data from field epidemiological studies (e.g. project 7.T.3 RHD Review) should be exploited to ask if there are rabbits seronegative to RHDV in breeding populations.

Considerations for action against rabbits

1. Genetic resistance to RHDV infection is apparently developing most rapidly in the warm, dry winter-rainfall areas of South Australia and north-western and Central Victoria as well as similar climatic areas in southern NSW. It is in areas with 300 – 600 mm annual average rainfall in southern Australia that additional resources for rabbit control (training, advice and availability of machinery and materials) will be most needed.

2. In general, an oral dose equivalent to $1500LD_{50}$ (0.5 ml of commercially available RHDV suspension) causes acute disease in most rabbits. Consequently, release of virus on baits or by inoculating field-caught rabbits should still cause acute infection, although direct intramuscular inoculation may be more reliable in areas where high levels of resistance become apparent.

3. Despite this, it must be recognized that Czech strain-351 virus might not spread as easily as was previously the case. Transmission of virus by flies, for example, would seem highly unlikely in areas where rabbits have high resistance to infection because fly spots contain few viral particles.

4. Contact with a small quantity of virus generally does not cause rabbits to develop antibodies that protect them against future bouts of RHD. A minority of rabbits do seroconvert without showing clinical disease but many rabbits simply do not become infected unless exposed to high virus dose. This means that most seronegative rabbits remain susceptible to high virus doses or new virus strains that evolve.

5. Although not explicitly discussed, the rate of evolution of resistance may be related to rabbit population density which is known to affect disease impact and population turn-over. This should be taken into account in considering integrated

rabbit control. For example, the possibility of retarding the development of resistance by keeping rabbits very low should be explored.

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References

1. Asgari, S., Hardy, J.R.E., Sinclair, R.G. and Cooke, B.D. (1998) Field evidence for the transmission of rabbit haemorrhagic disease virus (RHDV) by flies (Diptera: Callophoridae) among wild rabbits in Australia. *Virus Research*, 54, 123 – 132.
2. Best, S.M. and Kerr, P.J. (2000). Coevolution of host and virus: the pathogenesis of virulent and attenuated strains of myxomatosis in resistant and susceptible European rabbits. *Virology*, 267, 36 – 48.
3. Bruce, J.S., Twigg, L.E. and Gray, G.S. (2004). The epidemiology of rabbit haemorrhagic disease, and its impact on rabbit populations, in south-western Australia. *Wildlife Research* 31:31-49.
4. Capucci, L., Scicluna, M.T. and Lavazza, A. (1991). Diagnosis of viral haemorrhagic disease of rabbits and European brown hare syndrome. *Revue scientifique et technique de l'Office International des Epizooties*, 10, 347 – 370.
5. Capucci, L., Fusi, P., Lavazza, A., Pacciarini, M.L. and Rossi, C. (1996) Detection and preliminary characterization of a new calicivirus related to rabbit hemorrhagic disease virus but nonpathogenic. Journal of Virology 70, 8614-8623.
6. Capucci, L., Fallacara, F., Grazioli, S., Lavazza, A., Pacciarini, M.L. and Brocch, E. (1998). A further step in the evolution of rabbit haemorrhagic disease virus: the appearance of the first consistent antigenic variant. *Virus Research*, 58, 115 – 126.
7. Cooke, B.D. (2002). Rabbit haemorrhagic disease: field epidemiology and management of wild populations. *Revue scientifique et technique Office International des Epizooties*, 21, 347 – 358.
8. Cooke, B.D. and Berman, D. (2000). Effect of inoculation route and ambient temperature on survival time of rabbits, *Oryctolagus cuniculus* (L.), infected with rabbit haemorrhagic disease virus. *Wildlife Research* 27, 137 – 142.

9. Cooke,B. D., Chapuis,J.-L., Magnet,V., Lucas, A. and Kovaliski, J. (2004). Potential use of myxoma virus and rabbit haemorrhagic disease virus to control feral rabbits in the Kerguelen Archipelago. *Wildlife Research* 31, 415–420.
10. Cooke, B., Robinson, A.J., Merchant, J.C., Nardin, A. and Capucci, L. (2000). Use of ELISAs in field studies of rabbit haemorrhagic disease (RHD) in Australia. *Epidemiology and Infection* 124, 563 – 576.
11. Cooke, B.D. and Fenner, F. (2002). Rabbit Haemorrhagic Disease and the biological control of wild rabbits, *Oryctolagus cuniculus* (L.) in Australia and New Zealand. Wildlife Research, 29, 689 – 706.
12. Cooke, B.D. (1990) Notes on the comparative biology and laboratory breeding of the rabbit Flea *Xenopsylla cunicularis* Smit (Siphonaptera: Pulicidae). *Australian Journal of Zoology*, 38, 527 – 534.
13. Cooke, B. D., Robinson, A. J., Merchant, J. C., Nardin, A. and Capucci, L. (2000). Use of ELISAs in field studies of rabbit haemorrhagic disease (RHD) in Australia. *Epidemiology and Infection*, 124, 563 – 576.
14. Cooke, B. D., McPhee, S., Robinson, A. C. and Capucci, L. (2002) – Rabbit haemorrhagic Disease: Does a pre-existing RHDV-like virus reduce the effectiveness of RHD as a biological control agent in Australia? Wildlife Research 29, 673 – 682.
15. Dudzinski, M.L. and Mykytowycz, R. (1961). The eyelens as an indicator of age in the wild rabbit of Australia. *CSIRO Wildlife Research* 6, 156 – 159.
16. Fenner, F. and Fantini, B. (1999) Biological control of vertebrate pests. The history of myxomatosis – an experiment in evolution. CAB Publishing, Wallingford, Oxon, UK. 349 pp.
17. Fenner F. and Ratcliffe, F.N. (1965) *Myxomatosis*. Cambridge: Cambridge University Press.
18. Forrester, N.L., Boag, B. Moss, S.R., Turner, S.L., Trout, R.C., White, P.J., Hudson, P.J. and Gould, E.A. (2003). Long-term survival of New Zealand rabbit haemorrhagic disease virus RNA in wild rabbits, revealed by RT-PCR and phylogenetic analysis. *Journal of General Virology*, 84, 3079 - 3086.
19. Forrester, N.L., Trout, R.C., Turner, S.L., Kelly, D., Boag, B. Moss, S. and Gould, E.A. (2006). Unravelling the paradox of rabbit haemorrhagic disease virus emergence, using phylogenetic analysis; possible implications for rabbit conservation strategies. *Biological Conservation*, 131, 296 - 306.
20. Hall, E.J., Bremer, D.J., Worgul, B and Smilenov, L. (2005) Genetic susceptibility to radiation. *Advances in Space Research*, 35, 249 – 253.
21. Henzell, R. P., Cunningham, R. B. and Neave, H. M. (2002). Survival of Australian Rabbit populations exposed to rabbit haemorrhagic disease. Wildlife Research, 29, 523 – 542.
22. LeGall, G., Arnauld, C., Morrisse, J.P. and Rasschaert, D. (1998). Molecular epidemiology of rabbit haemorrhagic disease outbreaks in France during 1988 to 1995. *Journal of General Virology* 79, 11 – 16.
23. LeGall-Recule G, Zwingelstein F, Laurent S, de Boisseson C, Portejoie Y, Rasschaert D. (2003). Phylogenetic analysis of rabbit haemorrhagic disease virus in France between 1993 and 2000, and the characterization of RHDV antigenic variants. *Archives of Virology*, 148, 65 – 81.

24. Liu, G., Zhang, Y., Ni, Z Yun, T., Sheng, Z., Liang, H., Hua, J., Li, S., Du, Q. and Chen, J. (2006). Recovery of Infectious Rabbit Hemorrhagic Disease Virus from Rabbits after Direct Inoculation with In Vitro-Transcribed RNA. *Journal of Virology*, 80, 6597-6602.
25. Marshall, I. D. and Fenner, F. (1960). Studies in the epidemiology of myxomatosis. V. Changes in the innate resistance of Australian wild rabbits exposed to myxomatosis, *Journal of Hygiene,* 56, 288–302.
26. Moriarty, A., Saunders, G. and Richardson, B.J. (2000). Mortality factors acting on adult rabbits in central western New South Wales. *Wildlife Research*, 27, 613 – 619.
27. Mutze, G.J. (1996). Release of Spanish rabbit fleas as vectors of myxomatosis in inland South Australia. Australian Nature Conservation Agency, Canberra. Final Report of Project 24d, Feral Pests Program.
28. Mutze, G. Cooke, B. and Alexander, P. (1998). Initial impact of rabbit haemorrhagic disease on European rabbit populations in South Australia. *Journalof Wildlife Diseases*, 34, 221 – 227.
29. Nagesha, H.S., McColl, K.A., Collins, B.J., Morrissy, C.J., Wang, L.-F. and Westbury, H.A. (2000). The presence of cross-reactive antibodies to rabbit haemorrhagic disease virus in Australian wild rabbits prior to the escape of the virus from quarantine. *Archives of Virology*, 145, 749 – 757.
30. Neave, H.M. (1999). Rabbit Calicivirus Disease Program Report 1: Overview of effects on Australian rabbit populations and implications for agriculture and biodiversity. Bureau of Rural Sciences, Canberra.
31. Nix, H.A. (1986) A biogeographical analysis of Australian elaphid snakes In 'Atlas of Elaphid snakes of Australia'. (Ed. R. Longmore.) Australian Flora and Fauna Series No. 7 pp 4 – 15. (Bureau of Flora and Fauna: Canberra.)
32. Nowotny, N., Ros Bascuñana, C., Ballagi-Pordany, A., Gavier-Widen, D., Uhlen, M. and Belak, S. (1997). Phylogenetic analysis of rabbit haemorrhagic disease and European brown hare syndrome viruses by comparison of sequences from the capsid protein gene. *Archives of Virology***,** 142, 657 – 673.
33. O'Keefe, J.S., Tempero, J.E., Atkinson, P.H., Pacciarini, M.L., Fallacara, F., Horner, G. and Motha, J. (1998) Typing of rabbit haemorrhagic disease virus from New Zealand wild rabbits. New Zealand Veterinary Journal 46, 41-42.
34. Richardson, B.J. Phillips, S. Hayes, R.A. Lokanath S. and Cooke B.D. (in press). Aspects of the biology of the European rabbit (*Oryctolagus cuniculus*) and Rabbit Haemorrhagic Disease Virus (RHDV) in coastal eastern Australia. Wildlife Research 0, 000 – 000.
35. Rendel, J. M. (1971). Myxomatosis in the Australian rabbit population. *Search*, 2, 89-94.
36. Robinson, A. J., So, P. T. M., Müller, W. J., Cooke, B. D. and Capucci, L. (2002) Statistical models for the effect of age and maternal antibodies on the development of rabbit haemorrhagic disease in Australian wild rabbits. Wildlife Research 29, 663 – 671.
37. Ruvoen-Clouet, N., Ganiere, J.P., André-Fontaine, G., Blanchard, D., and LePendu, J. (2000). Binding of rabbit haemorrhagic disease virus to antigens of the ABH histo-blood group family. *Journal of Virology*, 74, 11950 – 11954.

38. Ruvoen-Clouet, N., Mas, E., Marrioneau, S., Gullion, P., Lombardo, D. and Le Pendu, J. (2006). Bile-salt stimulated lipase and mucins from milk of secretor mothers inhibit the binding of Norwalk virus capsids to their carbohydrate ligands. *Biochemical Journal*, 393, 627 – 634.
39. Saunders, G., Choquenot, D., McIlroy, J. and Packwood, R. (1999). Initial effects of rabbit haemorrhagic disease on free-living rabbit (*Oryctolagus cuniculus*) populations in central western New South Wales. *Wildlife Research*, 26, 69 – 74.
40. Sobey, W.R and Conolly, D. (1971) Myxomatosis: the introduction of the European rabbit Flea Spilopsyllus cuniculus (Dale) into wild rabbit populations in Australia. *Journal of Hygiene*, 69, 331 – 346.
41. Schirrmeier, H., Reimann, I., Kollner, B. and Granzow, H. (1999). Pathogenic, antigenic and molecular properties of rabbit haemorrhagic disease virus (RHDV) isolated from vaccinated rabbits: detection and characterization of antigenic variants. *Archives of Virology*, 144, 719 – 735.
42. Storey, G., Berman, D., Palmer, R. and Scanlan, J. (2004). The impact of rabbit haemorrhagic disease on wild rabbit (*Oryctolagus cuniculus*) populations in Queensland. *Wildlife Research*, 31, 183 – 193.
43. Thorven, M., Grahan, A., Hedlund, K-O., Johansson, H., Wahlfrid, C., Larson, G. and Svensson, L. (2005). A homozygous nonsense mutation (428G→A) in the human secretory (FUT2) gene provides resistance to symptomatic Norovirus (GGII) infections. *Journal of Virology*, 79, 15351 – 15355.
44. Williams, C.K., Moore, R.J. and Robbins, S.J. (1990) Genetic resistance to myxomatosis in Australian wild rabbits, ryctolagus cuniculus (L). Australian Journal of Zoology, 38, 697 – 703.
45. Williams, C.K., Parer, I., Coman, B.J., Burley, J. and Brasher, M.L. (1995) 'Managing Vertebrate Pests: Rabbits.' (Bureau of Resource Sciences and CSIRO Division of Wildlife and Ecology, Australian Government Publidhing Service: Canberra).