CENTRE FOR
INVASIVE SPECIES SOLUTIONS

# UNDERSTANDING RHDV2 INTERACTION WITH OTHER RHDVS AND ITS POTENTIAL AS AN ADDITIONAL RABBIT BIOCONTROL *and* NATIONAL RABBIT BIOCONTROL OPTIMISATION

Final Report for Projects P01-B-001 and P01-B-002

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We acknowledge the Traditional Custodians of the lands on which we meet and work and pay our respects to Elders — past, present and emerging.

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# UNDERSTANDING RHDV2 INTERACTION WITH OTHER RHDVS AND ITS POTENTIAL AS AN ADDITIONAL BIOCONTROL AGENT

# NATIONAL RABBIT BIOCONTROL OPTIMISATION

## FINAL PROJECT REPORTS FOR P01-B-001 AND P01-B-002

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# EXECUTIVE SUMMARY

The 2014 incursion of the rabbit haemorrhagic disease virus two (RHDV2) into Australia impacted the national release of the RHDV-K5 virus strain. RHDV2 quickly became the dominant circulating strain in Australia, likely favoured by its epidemiological advantages of being able to overcome immunity to existing strains and to fatally infect rabbits at a very young age. The latter made it appealing as a potential additional rabbit biocontrol tool as it was considered potentially suitable for land managers to release at any time of the year – including the breeding season. In addition, there was a clear need to increase our understanding of this new virus and its implications for rabbit biocontrol.

Between 2017 and 2022, two complementary CISS rabbit biocontrol projects investigated RHDV2 from every angle, with multiple subprojects to understand its biology, spread, diversity, evolution, impacts and interactions with other circulating viruses; assess its suitability as a potential additional registered biocide; and produce data needed for a future registration process with Australian Pesticides and Veterinary Medicines Authority (APVMA). Key components of this work included:

- virulence studies in domestic rabbits and a subsequent welfare assessment
- experimental studies assessing the effect of RHDV2 maternal immunity on RHDV2 infections in young rabbits
- experimental studies assessing the ability of RHDV2 to overcome existing immunity to other RHDVs (and vice versa, in experimentally infected domestic and wild-caught rabbits)
- ongoing long-term field studies to monitor rabbit abundance and their serological profiles at selected monitoring sites
- ongoing molecular testing of RHDV cases, including providing a free testing service for domestic-rabbit owners and for land managers wishing to identify the cause of wild-rabbit mortality
- genetic analysis to track virus evolution and adaptation, and reconstruct key epidemiological parameters and events from genetic sequences.

Key findings of these projects were:

- RHDV2 is highly virulent in adult and young domestic rabbits, with welfare outcomes similar to previously registered RHDVs.
- Maternal antibodies prevent lethal disease, but not infection, and lead to seroconversion in survivors, suggesting that year-round releases of a putative RHDV2 product are contraindicated.
- Experimental infections of both wild and domestic rabbits showed RHDV (including K5) was better able to overcome immunity to RHDV2 to infect rabbits than vice versa. This suggests that K5 may now be a better biocide compared to 2017 when it was initially released, because the 2017 population immunity to RHDV was fully protective against lethal K5 infection, and today's population immunity against RHDV2 is only partially protective.
- Serological monitoring at the long-term study sites as well as the serological profiles of the wild-caught rabbits used for challenge trials showed a high prevalence of RHDV2 antibodies (including short-lived antibody subclasses IgM and IgA) indicating frequent circulation of RHDV2 in the field.
- Serological and molecular monitoring confirmed RHDV2 as the dominant circulating virus in the field, although RHDV is still occasionally detected.
- As part of the free testing services provided to rabbit owners and land managers, a total of 1,908 samples were analysed between July 2017 and May 2022, yielding additional valuable

data on vaccination efficacy against RHDV-K5, and providing a platform to raise awareness and education around biological controls.

- Western Australia was the only state where we detected evidence that RHDV-K5 had established in wild rabbits and was circulating to some degree; all other K5 detections were associated with release sites.
- Periodic virus-sequencing analysis revealed multiple viral recombinants (naturally circulating viruses mixing and matching their genomes) that demonstrated increased epidemiological fitness, indicating that RHDV2 continues to evolve and adapt to Australian conditions, and highlighting the need for ongoing monitoring.
- The virus-sequence analysis also facilitated definition of key epidemiological parameters from genetic sequences that revealed insights into virus-interaction dynamics.
- Registered RHDV biocontrol products have potentially been released at the wrong time of the year (i.e. during the major rabbit-breeding seasons) up to 75% of the time. This potentially does significant damage to control efforts by introducing RHDV immunity into the wild-rabbit population and would be expected to make populations harder to control in the future. These results are of direct relevance to the possible registration of an RHDV2 biocide, which would also not be suitable for release when young rabbits are present in the population, and for which we would expect similar concerning patterns in virus releases by land managers.
- All project findings have been published in a total of 17 scientific articles in highly reputable peer-reviewed journals, with several more in preparation.

The combined results suggested there would be little benefit in registering RHDV2 as an additional biocide for local control at this point in time. This evidence-based recommendation is based on many project results/outcomes, with major factors including the high seroprevalence of RHDV2, its frequent natural-transmission events, and the attenuating effect from RHDV2 maternal immunity. This evidence-based recommendation is additionally supported by an independent economic assessment that found a negative return on investment ratio for an RHDV2 registered product. A final recommendation was therefore made by the CISS Rabbit Biocontrol Program Steering Committee to not proceed with the registration of RHDV2.

The combined project outputs also confirm the value of ongoing monitoring and evaluation of circulating biocontrol viruses, and highlight the possibility of substantial additional benefits from optimising the use of RHDV-K5 in the context of the RHDV2-dominant landscape. Recommendations going forward include optimising the formulation, usage and impacts of RHDV-K5; as well as better understanding its epidemiology after release and better integrating biocontrol with conventional controls. Such work would be supported by the development of improved ongoing serological and molecular testing; and monitoring and evaluation capacity.

# INTRODUCTION

Rabbits are a serious environmental and agricultural problem in Australia. They affect higher numbers of threatened animals and plants than any other invasive species in the country (Kearney et al. 2019). Their impacts include eating plants (direct herbivory) and the associated reduction in vegetation regenerating; competing for food resources; degrading land through reducing soil porosity (the space between particles of soil, important for air and water movement in soil), increasing the compaction and erosion of soil; allowing weed infestation; and supporting large populations of introduced predators (Finlayson, Taggart and Cooke, 2021). Many of these impacts occur at landscape scales, and some are even visible from space (Burrell, Evans and Liu, 2017; Pedler et al. 2016). Consequently, the economic impacts of rabbits have been estimated at approximately $200 million annually (Bradshaw et al. 2021; Cooke, Chudleigh, Simpson and Saunders, 2013; Gong, Sinden, Braysher and Jones 2009).

Viral biological controls have proven to be the most effective rabbit-management technique available because they are self-disseminating and act continuously at a continental scale. Two viruses have been used to assist in managing rabbits; (1) myxoma virus was introduced to Australia in 1950 and causes the disease myxomatosis; and (2) rabbit haemorrhagic disease virus (RHDV) was introduced to Australia in 1995 and causes rabbit haemorrhagic disease (RHD) (Cooke and Fenner 2002; Ratcliffe et al. 1952). Both viruses were highly effective at reducing rabbit populations following their introduction and they both now circulate naturally in rabbits in Australia (Mutze, Cooke and Alexander 1998; Ratcliffe et al. 1952).

Today, RHDV is the only virus that is available to land managers for local releases, but it is recommended that the virus is not released in the presence of young rabbits, which are innately resistant to lethal RHDV infection (Neave et al. 2018; Taggart et al. 2022b). In 2017, a variant of RHDV, RHDV-K5, was released nationwide to boost the effectiveness of RHDV-mediated biocontrol and to slow down the rate of recovery in rabbit populations (Strive and Cox 2019).

In 2010 a third virus, rabbit haemorrhagic disease virus 2 (RHDV2), emerged in France and subsequently spread across the globe (Rouco, Aguayo-Adán, Santoro, Abrantes and Delibes-Mateos 2019), including to Australia, where it was discovered in 2015 (Hall et al. 2015; Le Gall-Reculé et al. 2011). RHDV2 is able to overcome natural immunity to and vaccination against previous RHDVs and, unlike these, has the ability to lethally infect young rabbits as well as a series of other rabbit/hare (leporid) hosts, including the European brown hare (*Lepus europaeus*) (Dalton et al. 2012; Hall et al. 2017). RHDV2 spread rapidly across the Australian continent and largely replaced RHDV as the dominant virus circulating in wild rabbits in Australia (Mahar et al. 2018). In addition, RHDV2 interfered with the national release of the RHDV-K5 virus, which had previously been registered as an additional biocontrol/biocide tool to boost RHDV-mediated biocontrol (Strive and Cox 2019). Initial reports suggested that rabbit mortality due to RHDV2 was high; subsequent studies estimated average rabbit mortality to be 60% (Ramsey et al. 2020).

In Australia, this anecdotally high mortality rate from RHDV2 and its ability to lethally infect young rabbits made it appealing as a potential additional rabbit biocontrol tool. At the time, it was suggested that, if officially registered, RHDV2 may be suitable for land-manager release at any time of the year due to being capable of lethally infecting young rabbits. This would have given a registered RHDV2 product significant advantage over the currently registered RHDV-K5 product, which is recommended to not be released when young rabbits are present in the population.

This suggestion that RHDV2 be investigated as a new rabbit biocontrol also coincided with the increasing development of genetic resistance towards RHDV (Elfekih, Metcalfe, Walsh, Cox and Strive 2022; Elsworth, Kovaliski and Cooke 2012; Schwensow et al. 2017). Consequently, people interested in rabbit management at the time were eagerly looking for new options for rabbit control in Australia, and RHDV2 initially appeared to fit this description well.

In 2018, a five-year rabbit biocontrol program was then born out of the Centre for Invasive Species Solutions and packaged into two large projects to facilitate its delivery. While these projects differed in

their specific milestones, they were designed with a single, united objective – to increase the understanding of RHDV2 and its interactions with other circulating RHDVs, and in this context investigate if RHDV2 was suitable as an additional rabbit biocontrol to be officially registered and released by land managers for optimised biocontrol outcomes. These two projects covered a series of subquestions and studies that would investigate RHDV2 from all angles and generate the information necessary to officially register RHDV2 as a new, additional rabbit biocontrol tool that could be accessed by land managers. Broadly, the work areas of these two projects comprised a series of experimental infection studies designed to gather data necessary for the RHDV2 product registration process, as well as national epidemiological monitoring of the circulating biocontrol strains and their impact on wild populations, to gather essential background information and to estimate the potential benefit of a putative registered RHDV2 product.

# METHODS AND RESULTS

## TWO SEPARATE PROJECTS, ONE UNITED AIM

The official registration of new biocontrols and similar chemical products in Australia is overseen by the APVMA. The APVMA have set criteria that must be met and set information that must be provided to enable them to robustly assess new biocontrols or chemicals for registration and use in Australia. In addition, further experimental laboratory studies and field studies needed to be carried out to understand the properties, distribution and impact of the naturally circulating RHDV2, and to estimate benefits from an additional registered RHDV2 product.

## LABORATORY-BASED STUDIES (EXPERIMENTAL INFECTIONS)

Five major laboratory components in domestic rabbits were carried out: (1) assessment of RHDV2 virulence/effectiveness in domestic rabbits, (2) assessment of the humaneness of RHDV2 infection and disease in domestic rabbits, (3) assessment of the protection afforded by RHDV2 maternal antibodies to young rabbits, (4) support for the development of an RHDV2 vaccine (through an external company) for the protection of pet and commercial rabbits, and (5) assessment of the ability of RHDV2 to overcome existing immunity to other RHDVs (and vice versa) in domestic rabbits with known infection history. All of these major laboratory components have now been published in scientific journals, where we detail descriptions of the methodology of each component. We do not go into depth on methodology here.

### COMPONENT L1: ASSESSMENT OF RHDV2 VIRULENCE IN DOMESTIC RABBITS

*METHODS*

Hall et al. (2021a) used five-week-old rabbit kittens (18 male, 10 female) and 11-week-old adults (17 male, 11 female) from the disease-free captive-rabbit breeding colony at CSIRO Canberra for experimental RHDV2 challenge trials. All rabbits were confirmed seronegative to RHDV, RHDV2 and rabbit calicivirus Australia 1 (RCV-A1) prior to infection. Rabbits were housed individually, and orally inoculated with RHDV2 (GI.1bP-GI.2).

Rabbits received either a high (1,000 $RID_{50}$ [50% rabbit infectious dose]) or low dose (50 $RID_{50}$) of the challenge inoculum. They were monitored until humanely killed at a predefined humane end point (set to minimise unnecessary suffering) or for 10 days, whichever came first. Blood samples were collected from rabbits at numerous points throughout the study, and liver samples collected at the necropsy of each animal to confirm cause of death.

*RESULTS*

Hall et al. (2021a) observed a 100% case fatality rate in all rabbits irrespective of their age or infectious inoculum dose (Figure 1). However, rabbit kittens died quicker (mean: 39.3 hours post-infection) than did adults (mean: 52.5 hours post-infection), and adults that received a high dose of virus died quicker (mean: 46.8 hours post-infection) than adults that received a low dose of virus (mean: 58.2 hours post-infection).

The results confirmed that RHDV2 is highly virulent in naive domestic rabbits of all ages and provided data in support of a putative APVMA registration that requires the product to be highly effective for killing rabbits. This contrasts with previous reports of earlier RHDV2 isolates in Europe, where virulence was reported to be moderate or even absent in some cases (Calvete et al. 2018; Dalton et al. 2018; Le Gall-Reculé et al. 2013).

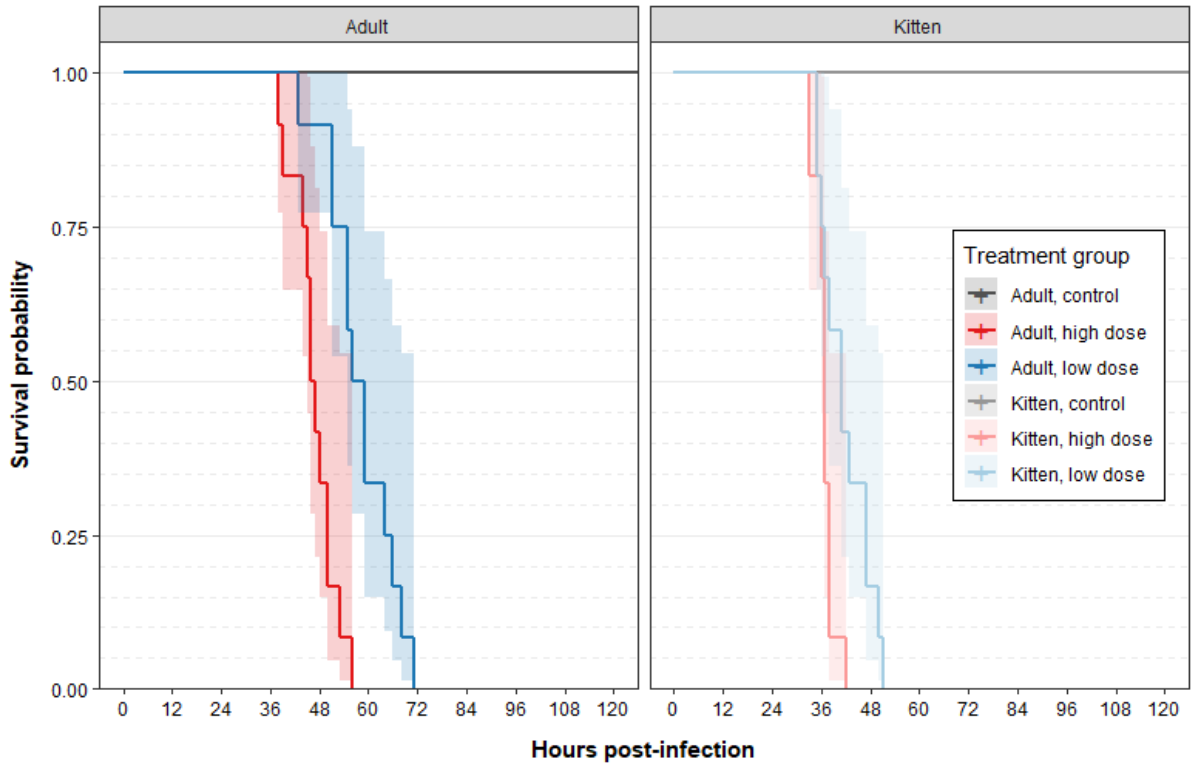


*Figure 1. Survival plot following RHDV2 challenge. Adult (11-week-old) rabbits and kittens (5-week-old) were challenged with either a high virus dose (1,000 $RID_{50}$; n = 12 per age) or a low virus dose (50 $RID_{50}$; n = 12 per age), or monitored as uninfected controls (n = 4 per age). The precise survival time after virus challenge was derived from continuous video-camera monitoring. Survival analysis was performed using the 'survminer' package in R. Transparent shaded areas represent 95% confidence intervals. Plots have been right-censored at 120 hours post-infection; all control animals survived until the end of the experiment (i.e. 10 days post-infection). Source: Hall et al. 2021a.*

## COMPONENT L2: ASSESSMENT OF THE HUMANENESS OF RHDV2 INFECTION AND DISEASE IN DOMESTIC RABBITS

*METHODS*

The assessment of the humaneness of RHDV2 infection and disease in domestic rabbits was also conducted by Hall et al. (2021a) through the same experimental challenge trials described in Component L1. To assess humaneness, researchers fitted all rabbits with accelerometers to track and monitor rabbit activity, and temperature loggers to track and monitor animal body temperature (Figure 2). Each rabbit was additionally continuously monitored throughout the trials using a video camera and underwent a full necropsy after death. Subsequently, the data generated through this component were presented at an RHDV2 welfare-assessment workshop, with panel members that included scientists and government and animal welfare (RSPCA) representatives. RHDV2 was assessed against the welfare-matrix model developed by Sharp and Saunders (2011) and included the welfare assessment for methods approved for the control of rabbits in Australia (Sharp 2020).

*RESULTS*

Hall et al. (2021a) reported rabbits that were inoculated with RHDV2 to experience fever (pyrexia), lethargy, weight loss and terminal seizures (immediately prior to and leading into death). Pyrexia was the most consistently observed clinical sign and developed between 23 and 48 hours post-infection in all infected rabbits. Time to the onset of pyrexia was shorter in kittens relative to adults, and in rabbits that were inoculated with a high virus dose. Lethargy was observed through both subjective monitoring and through objective activity monitors. In general, RHDV2-infected rabbits showed a decline in activity coincident with the onset of pyrexia, but most notably during the final hours of disease. Similarly, weight loss typically followed the onset of pyrexia, with infected kittens losing on

average three per cent and infected adults losing on average four per cent of body weight. Overall, the researchers suggest that welfare impacts of RHDV2 infection were experienced by kittens for 6–14 hours and by adults for 7–25 hours post-infection.

Seizures were observed in all infected animals that died and were characterised as intermittent episodes of generalised tonic-clonic seizure activity that commences between one and five minutes prior to death. All animals that died from terminal disease exhibited intermittent seizure activity for a period prior to death. Because blood glucose levels were significantly reduced at time of death, the seizures are presumed to be due to terminal hypoglycaemia as a result of terminal liver failure induced by the virus. While humans lose consciousness when experiencing tonic-clonic seizures, it remains unclear if the rabbits were conscious during or between seizures.

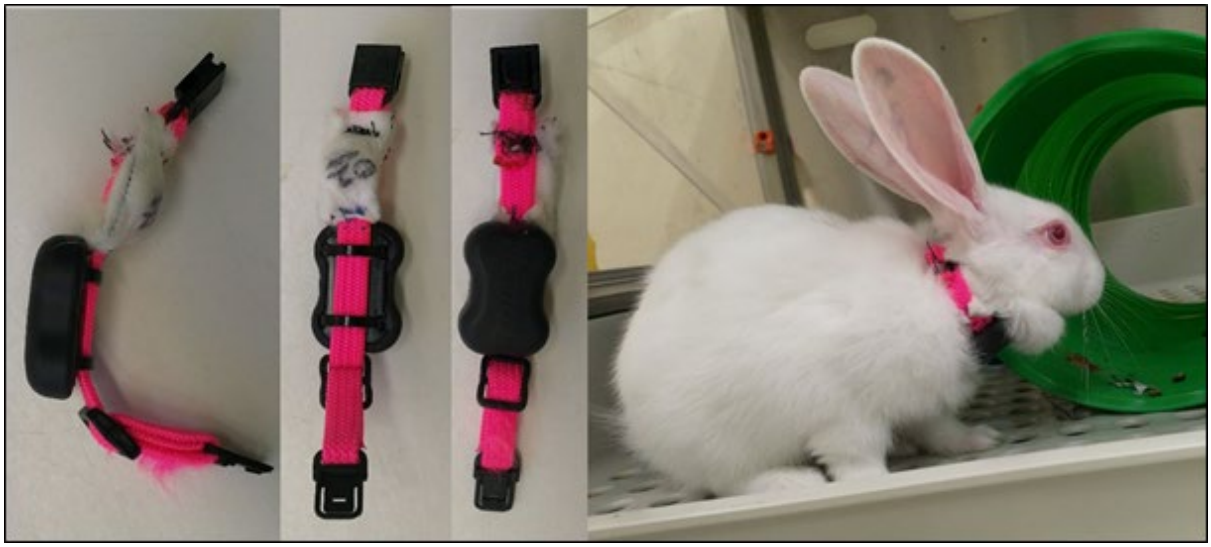

*Figure 2. Continuous temperature and activity monitoring of rabbits following RHDV2 challenge. A cat collar was fitted for each rabbit that comprised a SubCue-Mini temperature data logger within a fabric pouch and a 3D accelerometer to measure activity levels. The right panel shows the collar fitted on a rabbit. Source: Hall et al. 2021a.*

The welfare-assessment workshop for RHDV2 delivery on bait considered these results in the two-part assessment process that takes into consideration both the 'impact on the animal prior to the action that causes death' (Part A), as well as the 'actual mode of death' (Part B) and the extent and duration of suffering that is caused. With ingestion of baits there is usually little or no impact in Part A. Based on this assessment, the welfare impacts of RHDV2 were ranked similar to those of previous RDHV strains such as K5, and almost on-par with 1080 (the toxin sodium fluoroacetate) (Figure 3).

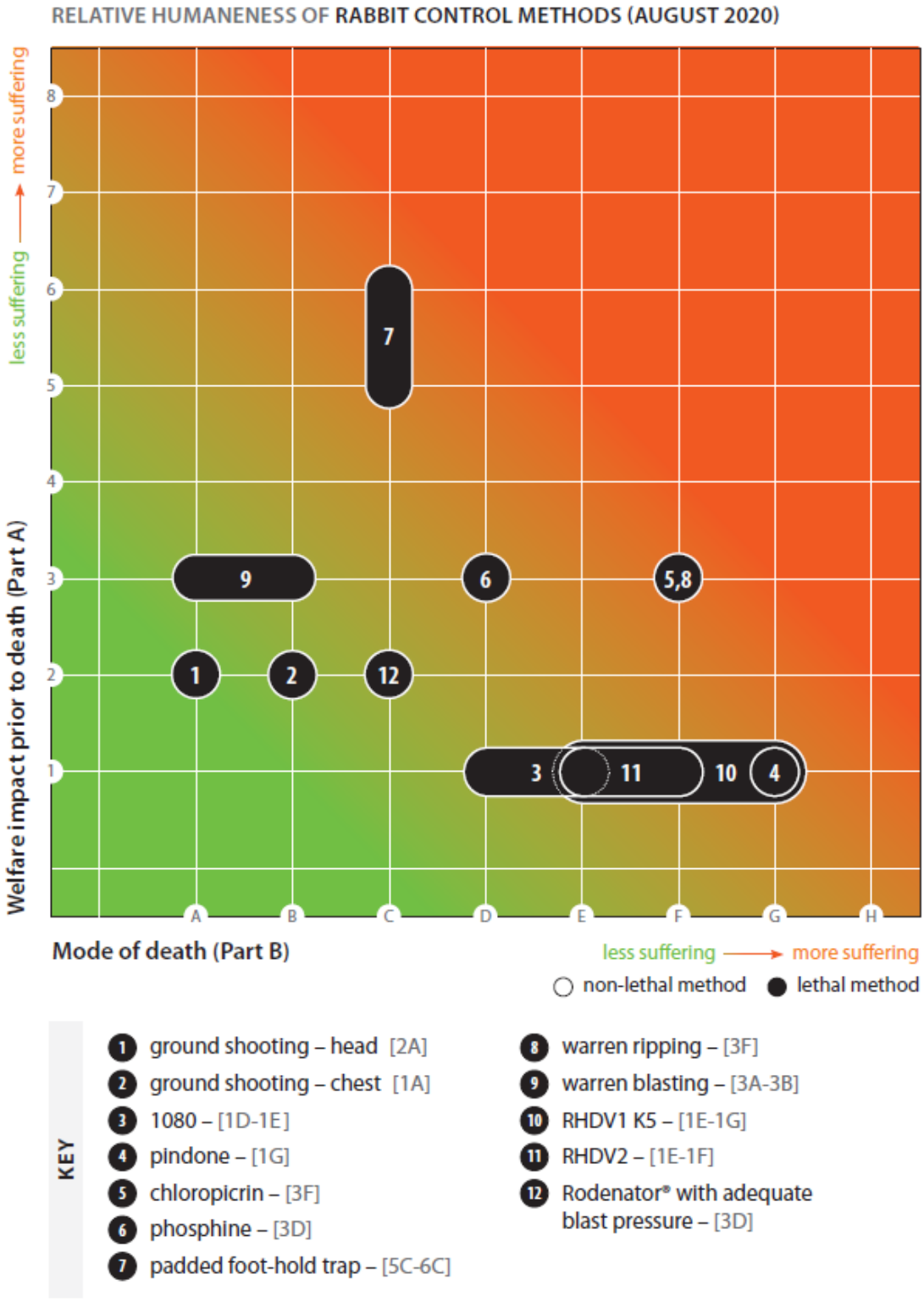


*Figure 3. Updated welfare matrix of different rabbit-control methods, including RHDV2 delivered on baits*

**COMPONENT L3: ASSESSMENT OF THE PROTECTION AFFORDED BY RHDV2 MATERNAL ANTIBODIES TO YOUNG RABBITS**

*METHODS*

The ability of RHDV2 to fatally infect rabbits at a very young age is unique among virulent rabbit caliciviruses (Neave et al. 2018) and led to the hypothesis that a registered product could be used year-round, including during the breeding season. This gives it a distinct advantage over the registered RHDV-K5 product that bears the risk of inadvertently vaccinating very young rabbits if released at the wrong time. However, in a landscape where RHDV2 has become the dominant circulating virus (Mahar et al. 2018), breeding does with acquired natural immunity to RHDV2 will pass RHDV2-specific passive maternal antibodies to their offspring. Their effect on RHDV2 infection in young rabbits therefore needed to be investigated, so a study was subsequently conducted by Hall et al. (2021b) for this purpose. Rabbit kittens of various ages were passively immunised with purified IgG antibodies to simulate maternal immunity (only antibodies of the IgG subclass cross the rabbit placenta and are detectable in kittens). Groups of four rabbits of 5, 7 and 9 weeks of age were injected with two doses (high and low levels of antibodies) and compared to non-immunised control groups. Twenty-four hours after immunisation these rabbits were challenged with a low dose of RHDV2 (50 $ID_{50}$), which was based on the estimated amount of virus a rabbit would consume on carrot bait. As in components L1 and L2, rabbits were euthanised at a humane end point or humanely killed after 10 days. Antibody titres were measured in the serum, and virus load was quantified in the liver at the end of the trial.

*RESULTS*

All rabbits treated with a high dose and 75% of those treated with a low dose of RHDV2 IgG to simulate maternal immunity survived the virus challenge. Surviving animals developed robust *virus*-specific antibody responses within 10 days post-infection (Hall et al. 2021b) (Figure 4). These findings demonstrate that the protection against RHDV2 conferred by passive immunisation is not sterilising. This is in contrast with previously published work for RHDV (Robinson, So, Müller, Cooke and Capucci 2002) where maternal immunity was sterilising in some cases, presumably due to the different route of inoculation used (intramuscular injection as opposed to the natural way of oral/mucosal infection used in this study). Sterilising immunity would have also been beneficial, as rabbits that do not become infected following a bait application can enter the pool of susceptible individuals once they have outgrown their passive maternal immunity and succumb during subsequent bait applications. However, when maternal immunity leads to attenuation of infection instead and results in seroconversion as observed here, this suggests that the presence of maternal antibodies in wild-rabbit populations may impede the effectiveness of RHDV2 as a biocontrol when young rabbits are present.

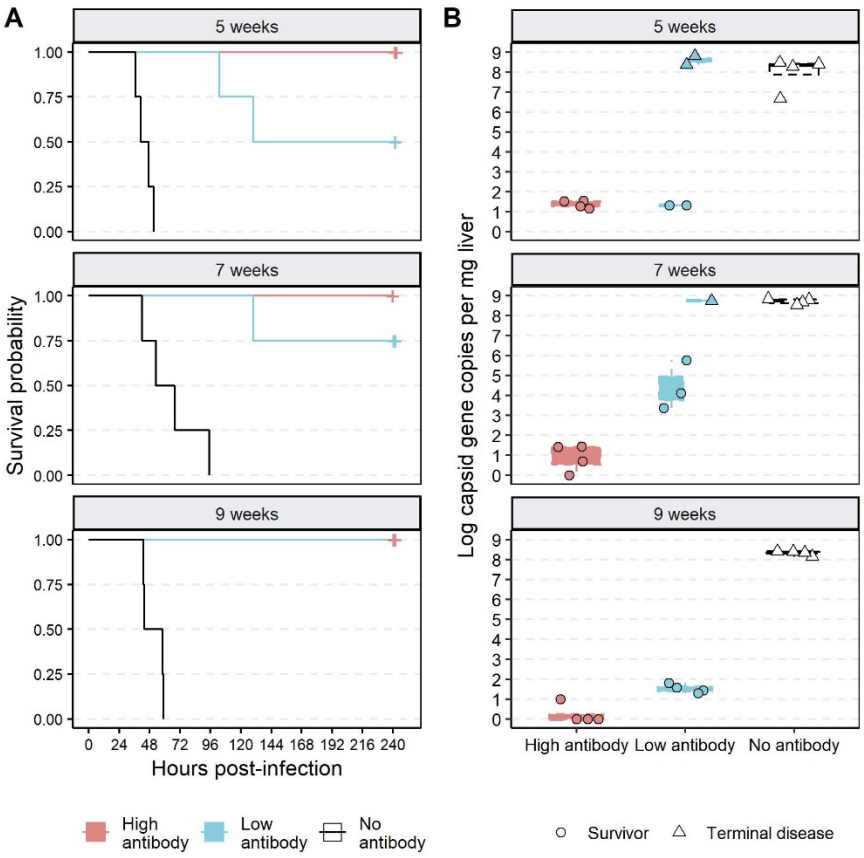


*Figure 4. Survival curves and viral RNA loads of passively immunised rabbits after RHDV2 infection. Rabbits aged five, seven or nine weeks old were passively immunised with either a high (pink) or low (blue) dose of RHDV2 IgG or PBS (white; control) by intramuscular injection in groups of four animals per treatment group. They were challenged 24 hours later with 50 $RID_{50}$ of RHDV2 and were monitored for the development of terminal rabbit haemorrhagic disease. (A) Survival time was obtained from continuous temperature monitors. Survival analysis was performed using the survminer package. (B) Total RNA was extracted from post-mortem liver samples and viral RNA was quantified by SYBR-based RT-qPCR. Individual data points and summary box plots are shown, coloured by dose of RHDV2 IgG. Triangles represent rabbits that developed terminal disease, while dots represent animals that survived infection (liver samples collected 10 days post-infection). Plots are faceted by age. Source: Hall et al. 2021b.*

## COMPONENT L4: DEVELOPMENT OF AN RHDV2 VACCINE FOR THE PROTECTION OF PET AND COMMERCIAL RABBITS

### *METHODS*

In support of a possible registration of RHDV2 as a product, the development of a vaccine by O'Connor et al. (2022) was conducted to protect non-target animals from any detrimental effects of a virus release; namely, pet and farmed rabbits. The ultimate aim was to produce a vaccine that would cover all RHDVs circulating in Australia (the original RHDV-Czech strain released in 1996, RHDV-K5 and RHDV2). However, to expedite the availability of a vaccine, the monovalent RHDV2 vaccine component was also investigated for its efficacy for a potential fast-tracked registration process. The individual vaccine components were produced from the respective virus strains as described previously (Abrantes and Lopes 2021; O'Connor et al. 2022). Groups of rabbits aged 10–12 weeks were immunised either with the multivalent or the monovalent RHDV2 vaccine and challenged 28 days later with the three different virulent viruses (RHDV2, RHDV-Czech and RHDV-K5) and survival was monitored.

*RESULTS*

All animals (*n* = 9) vaccinated with the monovalent vaccine survived the challenge with the homologous RHDV2 virus. The monovalent vaccine was not protective against the heterologous viruses (RHDV-Czech and RHDV-K5). All animals vaccinated with the multivalent vaccine were protected against lethal infection from all three viruses tested (Table 1).

*Table 1. Proportion of vaccinated rabbits that succumbed to RHD following oral inoculation with Australian lagoviruses. GI.4c = RCV-A1, GI.1 = RHDV (strain undetermined), GI.1a = RHDV-K5, GI.1c = RHDV-Czech, GI.2 = RHDV2, according to the nomenclature suggested in Le Pendu et al. (2017). Source: O'Connor et al. 2022.*

| Vaccination Status | Vaccine Dose (HAU [1]) | Challenge Virus (Dose 1500 $RID_{50}$) | Survived/Total |
|---|---|---|---|
| Seronegative | No vaccine | GI.1c | 0/12 |
| Seronegative | No vaccine | GI.1a | 0/12 |
| Seronegative | No vaccine | GI.2 [2] | 0/12 |
| GI.2 [2] | 128 | GI.1c | 0/6 |
| GI.2 [2] | 128 | GI.1a | 0/6 |
| GI.2 [2] | 100 | GI.2 [2] | 9/9 |
| Multivalent | 96 | GI.1c | 6/6 |
| Multivalent | 96 | GI.1a | 6/6 |
| Multivalent | 96 | GI.2 [2] | 6/6 |

[1] Total hemagglutination units. [2] This GI.2 refers to a RHDV2 recombinant designated GI.1bP-GI.2.

**COMPONENT L5: OVERCOMING NATURAL IMMUNITY TO HETEROLOGOUS RHDVS IN EXPERIMENTALLY INFECTED LABORATORY RABBITS (WITH KNOWN INFECTION HISTORY)**

*METHODS*

When using a putative registered product it is not only important to know that it is highly virulent in naive rabbits and to what degree it could be expected to overcome pre-existing immunity to other strains known to be present in Australia – including RHDV2, RHDV-Czech, RHDV-K5 and the benign endemic calicivirus RCV-A1. To this end, groups of animals (various sizes) were experimentally inoculated with the respective viruses, allowed to mount a strong immune response, then challenged with heterologous viruses to quantify the extent of cross-protective immunity. In some cases, groups of rabbits that were acquired from commercial breeders were used serendipitously for this component, as they had survived an unplanned outbreak of one of the naturally circulating viruses in their facility and were not usable for other studies (e.g. vaccine studies). The exact experimental designs are described in O'Connor et al. (2022).

*RESULTS*

O'Connor et al. (2022) observed some level of cross-protection against lethal challenge for all the heterologous viruses tested (Table 2). The degree of cross-protection varied greatly; it depended on the virus used, the infectious challenge dose, and the time that had passed between the first infection and the challenge. This is in line with previous observations on the transient nature of cross-protection conveyed by heterologous lagoviruses (Strive et al. 2013).

*Table 2. Immunity status, age at challenge, challenge virus, infectious dose and subsequent survival proportion of rabbits with experimentally or naturally acquired immunity. GI.4c = RCV-A1, GI.1 = RHDV (strain undetermined), GI.1a = RHDV-K5, GI.1c = RHDV-Czech, GI.2 = RHDV2, according to the nomenclature suggested in Le Pendu et al. 2017. Source: O'Connor et al. 2022.*

| Immunity Status | Age at Challenge (Weeks) | Challenge Virus | Infectious Dose ($RID_{50}$ [1]) | Survived/Total |
|---|---|---|---|---|
| Seronegative | 11 | GI.2 [2] | 50 | 1/8 [3] |
| Seronegative | 16 | GI.1a | 1500 | 0/12 |
| Seronegative | 16 | GI.1c | 1500 | 0/12 |
| Seronegative | 16 | GI.2[2] | 1500 | 0/12 |
| GI.4c | 11 | GI.2[2] | 50 | 7/9 |
| GI.1a | 11 | GI.2[2] | 50 | 7/7 |
| GI.1 [4] | 12 | GI.2[2] | 150 | 9/10 |
| GI.1 [4] | 12 | GI.2[2] | 1500 | 5/9 |
| GI.1 [4] | 33 | GI.2[2] | 1500 | 1/7 |
| GI.1 [4] | 33 | GI.1a | 1500 | 7/7 |
| GI.2 | 10–12 | GI.1a | 1500 | 1/4 |
| GI.2 | 10–12 | GI.1c | 1500 | 2/3 |
| GI.2 | 10–12 | GI.2[2] | 1500 | 3/4 |
| GI.2 [5] | 12 | GI.1a | 150 | 9/12 |
| GI.2 [5] | 12 | GI.1a | 1500 | 9/13 |
| GI.2 [5] | 12 | GI.2 [2] | 150,000 | 12/12 |

[1] The 50% rabbit infectious dose ($RID_{50}$). [2] This GI.2 refers to a naturally occurring RHDV2 recombinant virus designated GI.1bP-GI.2 (GenBank acc. Number MW467791). [3] This one surviving rabbit did not seroconvert after challenge and was not infected with GI.2. [4] These rabbits could have antibodies from either GI.1a or GI.1c exposure. [5] This GI.2 refers to a naturally occurring RHDV2 recombinant virus designated GI.4cP-GI.2 (GenBank acc. Number MW460156).

## FIELD EPIDEMIOLOGY–BASED STUDIES

Multiple, major field-epidemiology project components were carried out to provide the essential understanding of which virus is where, when, and how they interact with each other. This epidemiological data also provided important underpinning data for the assessment of RHDV2 as a potential additional registered biocontrol. These multiple field-epidemiology components were:

1. monitoring the viruses responsible for rabbit deaths across the country by providing free molecular testing of rabbits found dead, and assessing their genetic diversity and evolution
2. using the genetic data to infer epidemiological parameters for both RHDV and RHDV2
3. conducting ongoing, long-term monitoring of selected rabbit populations (antibody status and rabbit abundance) across Australia to assess the infection dynamics and impacts of circulating viruses
4. assessing the cross-protection afforded by opposing RHDV/RHDV2 viruses in wild-caught rabbits with inferred (but unknown) infection history.

In addition, the project resources were used to carry out additional analyses on the accumulating epidemiological data from these projects and the past RHDV Boost project to analyse: (5) changes in the outbreak dynamics in an RHDV2-dominant landscape, and (6) an analysis of the timing of bait applications based on the available RHDV-Czech and K5 purchasing history. Lastly, a seventh project component also assessed the suitability of using blowfly sampling to make additional inferences from the national monitoring of RHDV epidemiology. Most of these components have been published in scientific journals; consequently, we do not go into depth on methodology here. For detailed descriptions of the methodology of each component, please refer to the published article.

**COMPONENT F1: MOLECULAR EPIDEMIOLOGY: FREE SAMPLE-TESTING SERVICE AND GENETIC ANALYSES TO DETERMINE VIRUS ACTIVITY, DISTRIBUTION, GENETIC DIVERSITY AND EVOLUTION**

*METHODS*

This component tested dead-rabbit samples submitted for RHDV testing through the RabbitScan mobile and web application (Invasive Animals Ltd. 2020). This functionality within RabbitScan to request test kits and submit dead-rabbit samples for RHDV testing was originally established to track viruses before and after the RHDV-K5 release, but was then continued for the purposes of monitoring and developing a better understanding of virus activity, distribution, genetic diversity and evolution. In addition to samples obtained through RabbitScan, members of the public also contacted the CSIRO rabbit team directly to report dead rabbits and request a sampling kit for RHDV testing (https://research.csiro.au/rhdv/testing/). These samples were initially screened with a broadly reactive *Lagovirus* PCR, and if positive, the strain was determined with a number of strain-specific diagnostic methods (Hall et al. 2018; Mahar et al. 2018; Mahar et al. 2021). Results were collated and reported on a monthly basis for upload onto the RabbitScan disease tracker webpage (https://www.feralscan.org.au/rabbitscan/map.aspx?mapMode=rhdv) and circulated to interested stakeholders within government agencies and Wildlife Health Australia. Representative samples from over the last seven years were selected for whole-genome sequencing to track the molecular epidemiology and evolution, and to provide data for Component F2.

*RESULTS*

Between 2015 – when this free testing service was first established to facilitate data collection and disease tracking in preparation for the RHDV-K5 release and following the RHDV2 incursion into Australia – and May 2022, a total of 2,632 samples from dead rabbits were received and tested for the different RHDVs known to circulate in Australia. This testing scheme has resulted in a valuable network of sample submitters and provided essential epidemiological and genetic data. The data has enabled researchers to track the spread of various RHDVs across the continent, the proportion of case fatalities that can be attributed to the various strains (Figure 5), as well as the evolution and emergence of recombinants.

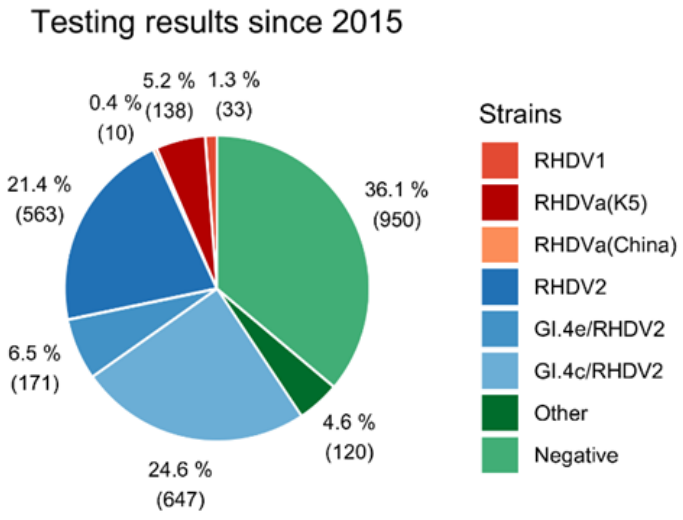


*Figure 5. Cumulative test results since the detection of RHDV2 in Australia (in 2015) until May 2022. Combined results from domestic and wild rabbits are shown. GI.4e/RHDV2 and GI.4c/RHDV2 depict recombinant viruses between RHDV2 and other circulating lagoviruses. 'Other' depicts samples that tested positive for multiple viruses or myxoma virus (not routinely tested for).*

During the duration of this project (between July 2017 and May 2022) a total of 1,908 samples were analysed; 1,410 were direct submissions and 498 via the Rabbitscan App. A substantial proportion of submitted samples from rabbits found dead test negative to all lagoviruses known to be present in

Australia. A separate project (Meat and Livestock Australia/CSIRO co-funded) has analysed some of these negative samples using next-generation sequencing approaches to identify possible causes of death. The *Clostridium* spp.*, Pasteurella multocida, Pseudomonas* spp. and *Eimeria stiedae* in some of these calicivirus-negative samples are all known to infect rabbits and are capable of causing fulminant disease (Jenckel, Hall and Strive 2022 preprint).

Domestic rabbits constituted the largest proportion of rabbit samples submitted for testing. However, many of the samples submitted through RabbitScan lacked information on domestic or wild origin of rabbits. As RabbitScan is the preferred submission pathway for landholders (direct submission is the preferred avenue for pet owners or rabbit breeders), it is likely that a large proportion of the rabbits from 'unknown origin' are wild rabbits. For many (but not all) of the domestic-rabbit sample submissions, vaccination histories were available. To date, we have not detected any rabbits vaccinated with the currently available vaccine that tested positive for RHDV-K5.

Many of the testing requests from domestic-rabbit owners and farmers were accompanied by requests for advice on how to manage biosecurity risks related to rabbit biocontrol agents. With several hundred testing requests every year, this provided a valuable community engagement platform to help educate members of the public on all aspects of rabbit biocontrol. Information we provided included the number and type of biocontrol viruses (RHDVs and myxoma) present in Australia, which ones are used for deliberate releases and which ones circulate naturally, what type of vaccines are available and what they protect against, how the various viruses can be transmitted, what biosecurity measures can be implemented to protect pet and farmed rabbits from naturally circulating biocontrol viruses, and how to decontaminate premises and pet enclosures following an outbreak. Providing practical information and guidance to submitters who were often emotionally and/or financially affected by the loss of their animals was an important and powerful avenue to increase public awareness and education on rabbit biocontrol.

Sequencing analysis of selected strains revealed several recombinant virus derivatives of RHDV2. Recombinant viruses can emerge when two different strains of rabbit caliciviruses infect the same host at the same time and their respective genomes form a chimera. If there is an epidemiological advantage in the newly acquired combination of genes, these recombinants can be selected for. This has been observed at least six times independently between RHDV2 and other circulating rabbit caliciviruses in Australia so far, including non-pathogenic RCV-A1 viruses (Mahar et al. 2021). All recombinants carry the capsid gene of RHDV2 and are virulent. Figure 6 illustrates that following their emergence, these recombinants became locally dominant and replaced previous versions of RHDV2 due to an epidemiological-fitness advantage.

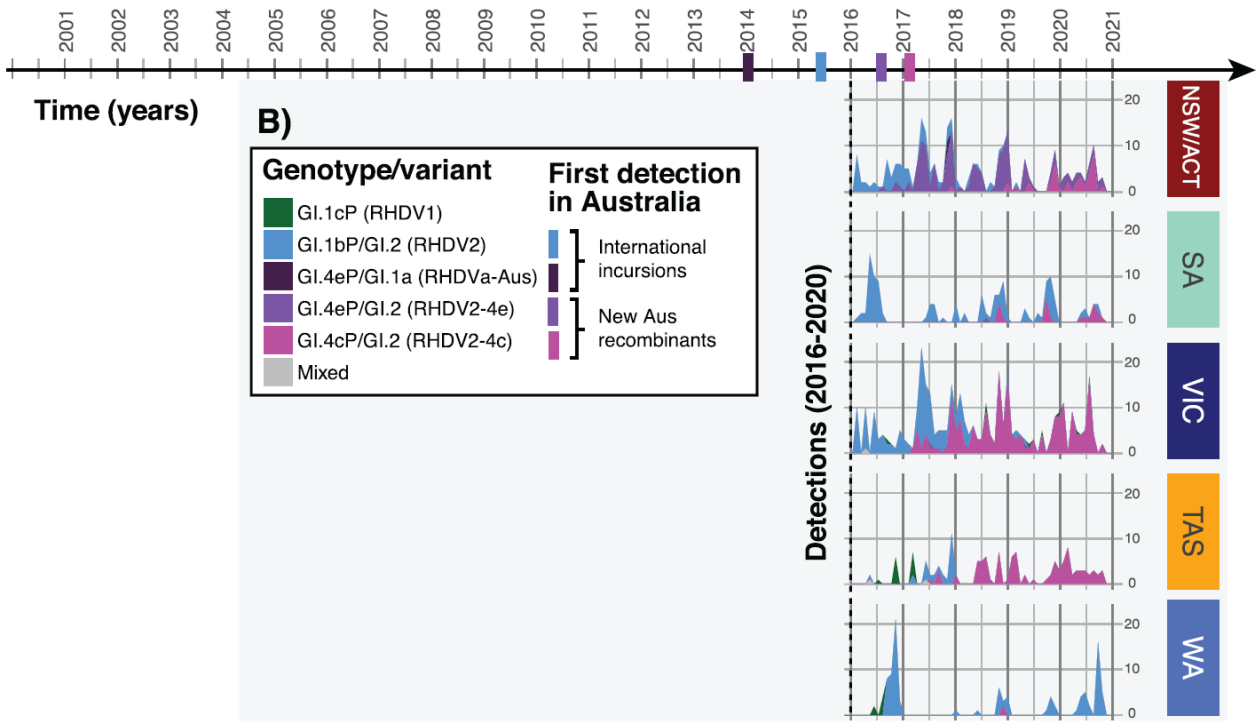


*Figure 6. Emergence and local spread of RHDV2 recombinants. Lagovirus-positive samples collected in NSW/ACT, SA, Vic, Tas and WA from 2016 to 2020 (n = 739) were genotyped to the variant level by sequencing either side of the typical calicivirus recombination breakpoint. The number of detections of each variant by month are shown for each geographical region as an area plot, with the plotted area coloured by variant. Source: Mahar et al. 2021.*

RHDV2 and its recombinants continue to be epidemiologically dominant in Australian wild and domestic rabbits; however, RHDV1 has not yet become completely extinct and is occasionally detected. K5 detections have decreased since the national rollout in 2017 and were mostly associated with virus releases. An interesting exception to this is WA, the only state where K5 cases were detected that were not associated with deliberate virus releases. This is determined by sequence analysis – K5 that has circulated multiple times through wild rabbits has accumulated some genetic changes as part of its natural evolution, whereas experimentally released viruses are genetically near-identical. Of note is also the pattern of positive RHDV2 detections. Although this type of sampling is opportunistic rather than systematic, a seasonal pattern is emerging with an increase in submissions around spring, possibly suggesting increasing disease activity. This notwithstanding, positive RHDV2 detections occurred every single month since the testing commenced in 2015, suggesting some level of year-round disease activity.

## COMPONENT F2: INFERRING EPIDEMIOLOGICAL PARAMETERS FROM VIRAL GENETIC SEQUENCES

*METHODS*

Among newly developed approaches to analyse genetic data, phylodynamic models show potential to reveal changes to viral populations over short periods and determine important epidemiological parameters such as the effective reproductive number (Re) – the average number of secondary infections per each infectious case. The release or incursions of the various RHDVs followed by their spread across the continent provides a unique dataset with which to investigate such phylodynamic models. Initially, the method was validated using the release and spread of the first RHDV1 in 1996, with detailed methods described in the resulting publication (Pacioni et al. 2022). Subsequently, these were applied to the larger genomic dataset acquired during the initial spread of RHDV2 across Australia between 2014 and 2020 (Pacioni et al. in review) to investigate the epidemiology of various strains, use molecular data to date the emergence of new variants and evaluate whether different viruses are (out)competing one another.

*RESULTS*

The pilot study carried out on published RHDV sequences revealed that this type of genomic data is suitable to infer epidemiological parameters (Pacioni et al. 2022), although the relative lack of available sequence information for the period covered posed challenges. Applying these methods to the more recent and abundant genetic data on RHDV2 acquired during this project, we aimed to investigate the epidemiology of various strains, to date the emergence of new variants and evaluate whether different viruses are (out)competing one another. The analysis showed that the two main RHDV variants in Australia (RHDV1 and RHDV2) had similar dynamics after their release, although over substantially different time frames (substantially shorter for RHDV2). We also found a strong geographic difference in between the two viruses as well as evidence of overall competition between them. This data has been submitted for publication and is currently undergoing peer-review (Pacioni et al. in review).

**COMPONENT F3: ONGOING, LONG-TERM MONITORING OF SELECTED RABBIT POPULATIONS (ANTIBODY STATUS AND RABBIT ABUNDANCE) ACROSS AUSTRALIA TO ASSESS THE INFECTION DYNAMICS AND IMPACTS OF CIRCULATING VIRUSES**

*METHODS*

Throughout the course of this five-year project, quarterly rabbit-biocontrol monitoring has occurred at one site in the ACT, one site in NSW, two sites in SA and two sites in WA. These monitoring events involve three consecutive nights of rabbit spotlight counts and a fourth consecutive night where up to a maximum of 20 rabbits are shot. Shot rabbits are subject to necropsy; and the collection of blood, tissue and an eyeball for serological analysis to test for exposure to circulating rabbit biocontrol viruses, genetic studies and rabbit aging, respectively. This serological data facilitates the investigation of biocontrol seroprevalence and dynamics. Detailed methods of the quarterly rabbit monitoring are described in Taggart et al. (2022a) and Ramsey et al. (2020), who also describe RHDV2 serological dynamics to some extent.

In addition to the quarterly rabbit-biocontrol monitoring, another study site (Turretfield in SA) is monitored at high frequency. The monitoring of this site involves approximately eight-weekly field trips where rabbits are captured in cage traps, blood and tissue samples collected, rabbits are tagged and then released. This study site represents a long-term capture–recapture site that offers longitudinal rabbit data at a much higher resolution than all other monitoring that occurs across Australia.

*RESULTS*

The number of national rabbit-monitoring sites declined substantially following the end of the RHDV Boost program in 2017. At the end of 2017, 24 sites were monitored as part of the national rabbit-biocontrol monitoring program. For the two CISS rabbit projects, one site each in the ACT and NSW continued, and two sites in both SA and WA. The number of ongoing sampling sites over time and their sampling frequency are indicated in Figure 7. During the early phase of this project there was some lag time before some of the six ongoing sites ‘came online’ again, resulting in gaps in the data for 2018. Furthermore, the summer 2019/2020 bushfires and COVID-19 affected sampling at some sites. While sampling continued at the ACT site between winter 2020 and winter 2021, rabbit numbers were too low to obtain a large enough sample to include in the analysis; these time points are shown as ‘not sampled’ in this report. No summer sampling occurred in SA in January 2022.

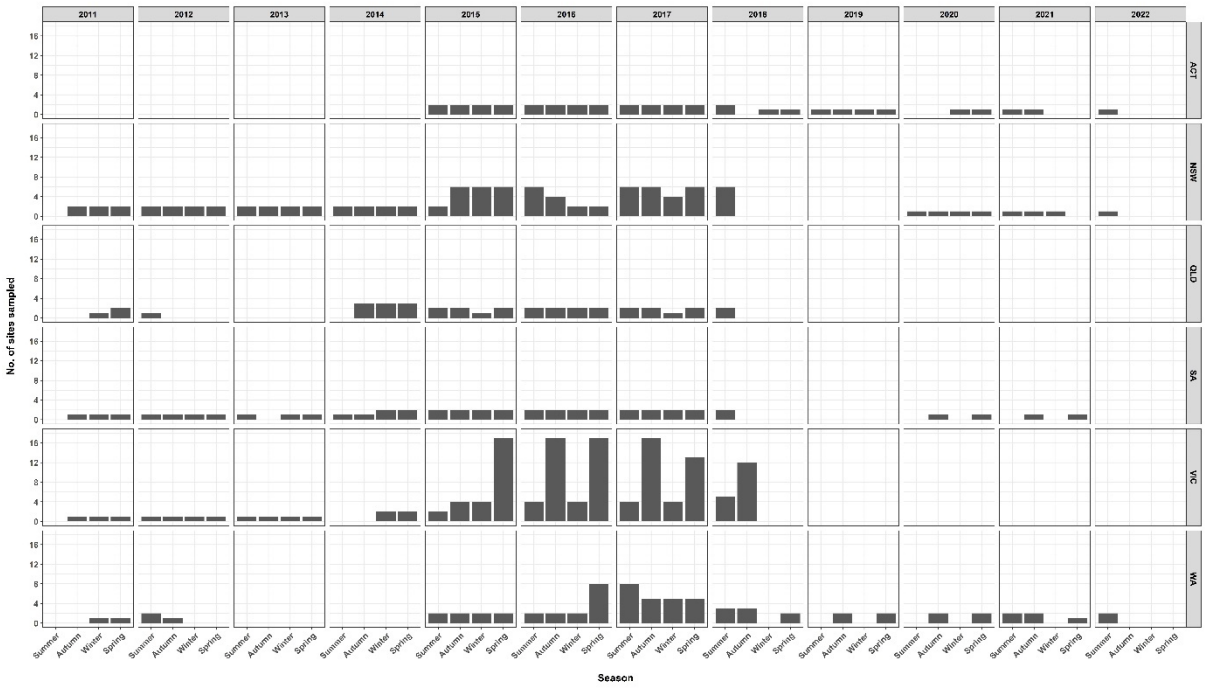


*Figure 7. Number of sites sampled in various Australian states and territories from 2011 to summer 2022 through the previous RHDV-Boost and Boost Rollout projects and the current CISS project P01-B002. This graph does not include the Turretfield site. Source: Kandarp Patel.*

All serum samples were tested using seven different antibody tests, the purpose and limitations of which are outlined in Table 3. The development and/or adaptations for these assays for RHDV2 is described in Strive et al. (2020).

*Table 3. Different serological assays for the long-term field-monitoring sites*

| Assay | Titrated out | Specificity | Sensitivity | Purpose |
|---|---|---|---|---|
| RHDV-2 cELISA | Yes | High | Moderate | Presence of RHDV2 antibodies, if the titre is higher than in those in the RHDV-1 cELISA |
| RHDV-1 cELISA | Yes | Moderate | Moderate | Presence of RHDV1 antibodies, if the titre is higher than in the RHDV-2 cELISA |
| RCV-A1 bELISA | Yes | Very high | Moderate | Presence of antibodies to RCV-A1 |
| RHDV1 IgG | Yes | Very low | Very high | Changes in titres of antibodies to almost any calicivirus. Also used to infer maternal immunity |
| RHDV2 IgM | No<br>1:40 only | Low | High | Presence of short-lived IgM antibodies indicates very recent exposure of the population to RHDV1 or RHDV2 |
| RHDV2 IgA | No<br>1:40 only | Low | High | Increase in IgA prevalence indicates very recent exposure of the population to RHDV1 or RHDV2 |
| Myxo | No<br>1:100 only | n/a | n/a | Presence of antibodies to myxomavirus |

A previous study by Ramsey et al. (2020) found that, based on serological data until January 2018, RHDV2 arrived in Australia in 2014 and rapidly spread across the country within two years. Following the establishment of RHDV2, wild-rabbit populations were reduced by an average of 60%, with impacts most pronounced in South and Western Australia. RHDV2 was reported to have negatively impacted on the ability of RHDV and RCV-A1 to spread within Australia, leading to a decrease in the prevalence of both viruses.

More recently, the virus prevalence data for RHDV2, RHDV1 and RCV-A1, as well as the spotlight-count data from the ongoing sites between 2018 and 2022, were added to the existing models used by Ramsey et al. (2020). This enabled the re-assessment of trends in seroprevalence and rabbit numbers for the more recent 2018–2022 period (Ramsey et al. in preparation).

Average trends in seroprevalence from the six sites with post-2018 serum samples revealed that RHDV seroprevalence continues to decline, although there was some serological evidence for recent RHDV transmission – especially at the SA and WA sites – suggesting that despite its decline it has not yet become completely extinct, which is in line with the molecular testing described in Component F1. In contrast, RHDV2 seroprevalence continued to increase, with an average adult seroprevalence of approximate 60–70%, confirming it as the dominant virus in the landscape. In contrast to RHDV, the seroprevalence of RCV-A1, while declining initially, appears to be making a recovery with average RCV-A1 seroprevalence in both juveniles and adults increasing over the last two years. Similarly, RHDV2 seroprevalence at the longitudinal Turretfield study site showed a rapid rise in seroprevalence after RHDV2 arrival and that seroprevalence is now being maintained at approximately 40%.

Inclusion of the available rabbit-abundance data from 2018 to 2022 into the models from Ramsey et al. (2020) suggests that RHDV2, for now, continues to suppress wild populations by an average of 64% compared to levels before its arrival.

**COMPONENT F4: ASSESSMENT OF CROSS-PROTECTION AFFORDED BY OPPOSING RHDV/RHDV2 VIRUSES IN FIELD-CAUGHT WILD RABBITS**

*METHODS*

Patel et al. (2022) captured 200 wild rabbits from five sites around the Adelaide region (within 50 km of the city) to assess the cross-protection afforded by opposing RHDV/RHDV2 viruses. Trapped rabbits were individually housed in insect-proof cages and transported back to an animal-housing facility. Rabbits were allocated to inoculation groups based on their capture serology where it was available prior to inoculation. For example, rabbits that were seropositive to RHDV2 at capture were either allocated to the RHDV-Czech or RHDV-K5 inoculation groups to assess to what extent RHDV-Czech/RHDV-K5 could overcome and cause disease or mortality in wild rabbits with natural RHDV2 immunity. Rabbits were orally inoculated with 1 mL of reconstituted RHDV-K5 or RHDV2 (following the manufacturer's instructions) and monitored until a predefined humane end point or for seven days after inoculation, whichever came first. Blood samples were collected from all rabbits at capture, immediately prior to inoculation and at death to test for exposure to RHDV, RHDV2 and RCV-A1. All rabbits were subject to necropsy examination after death, and liver samples collected for quantification of RHDV/RHDV2 using RT-qPCR.

*RESULTS*

Patel et al. (2022) found that in RHDV/RHDV2-seronegative rabbits at capture, infection rates were highest in those inoculated with RHDV2 (81.8%, 18 of 22 rabbits), followed by K5 (53.8%, 7/13) and Czech (40.0%, 2/5), but these differences were not statistically significant. In rabbits with previous exposure to RHDV2 at capture, infection rates were highest when inoculated with K5 (59.6%, 31/52) followed by Czech (46.0%, 23/50), with infection rates higher in younger rabbits for both viruses (Figure 8). In RHDV/RHDV2-seronegative rabbits at capture, case fatality rates were highest for those inoculated with K5 (71.4%), followed by RHDV2 (50.0%) and Czech (50.0%). In rabbits with previous exposure to RHDV2 at capture, case fatality rates were highest in rabbits inoculated with K5 (12.9%) followed by Czech (8.7%), with no case fatalities following RHDV2 inoculation. Case fatality rates did not differ significantly between inoculums in either serostatus group at capture. The overall

seroprevalence to RHDV2 of rabbits at capture was high; a very high proportion of rabbits (64.3%) showed serological evidence for a recent infection (or re-infection).

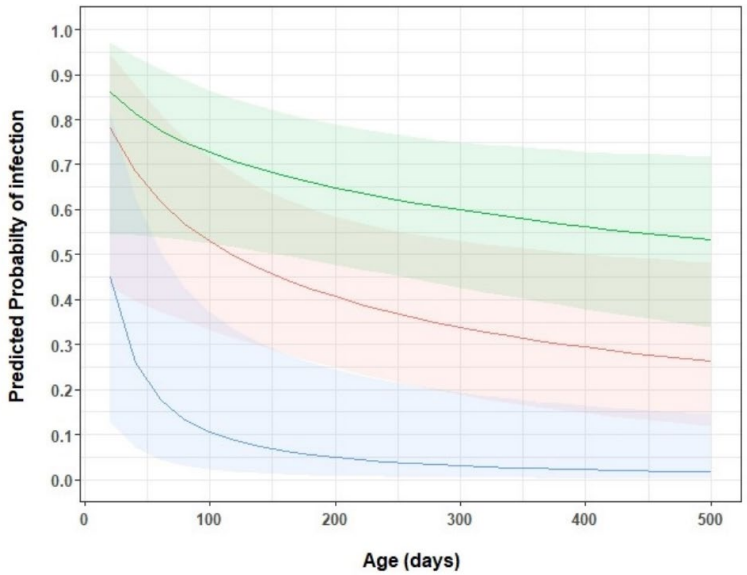


*Figure 8. Predicted infection probabilities following inoculation with Czech (red), K5 (green) and RHDV2 (blue) by age (in days) in rabbits with evidence of recent RHDV or RHDV2 infection at capture. Shaded areas represent the 95% confidence interval for their respective estimates. Source: Patel et al. 2022.*

**COMPONENT F5: FREQUENCY OF RHDV2 TRANSMISSION**

*METHODS*

Although their study was not designed with this purpose in mind, the analysis and data presented in Taggart et al. (2022a) enable us to interpret the frequency of RHDV2 transmission to some extent, due to the use of short-lived antibodies of the IgM and IgA subclass representing recent virus transmission. Taggart et al. (2022a) used a long-term serological dataset spanning the emergence and establishment of RHDV2 at 12 sites throughout Australia to examine any differences in the epidemiology compared to RHDV. Specifically, their study investigated patterns in short-lived IgA and IgM antibodies within an RHDV-dominant landscape (prior to the emergence of RHDV2) and within an RHDV2-dominant landscape (after the emergence of RHDV2).

*RESULTS*

Taggart et al. (2022a) used serological markers of recent RHDV/RHDV2 infection (IgM and IgA) and spotlight-count data from 2011 to 2018 from 12 rabbit-monitoring sites across Australia to investigate if and how the arrival of RHDV2 changed virus transmission dynamics. Following the arrival of RHDV2, they found that seasonal peaks in IgM and IgA seropositivity shifted forward one season, from winter to autumn and spring to winter, respectively (Figure 9). Contrary to predictions, they also found only weak effects of rabbit age, seropositivity to non-pathogenic calicivirus RCV-A1, and population abundance on IgM/IgA seropositivity. From these findings they concluded that RHDV2 enters rabbit populations shortly after the commencement of annual breeding cycles. Upon entering, the population RHDV2 undergoes extensive replication in young rabbits, causing clinical disease, high virus shedding, mortality and virus-laden carcasses. This results in high virus contamination in the environment, furthering the frequent transmission of RHDV2 and initiating outbreaks, while simultaneously removing the susceptible cohort required for the effective transmission of RHDV. Although RHDV may enter the population at the same time point, it is subclinical in young rabbits, causing minimal virus shedding and low environmental contamination. Their results demonstrate a major shift in epidemiological patterns in virus transmission by providing the first evidence that RHDV2's ability to clinically infect young rabbits is a key competitive advantage in the field. Their results additionally point to the ability of RHDV2 to enter rabbit populations rapidly after the birth of new, susceptible rabbit kittens: hence its ability to transmit more frequently relative to RHDV viruses.

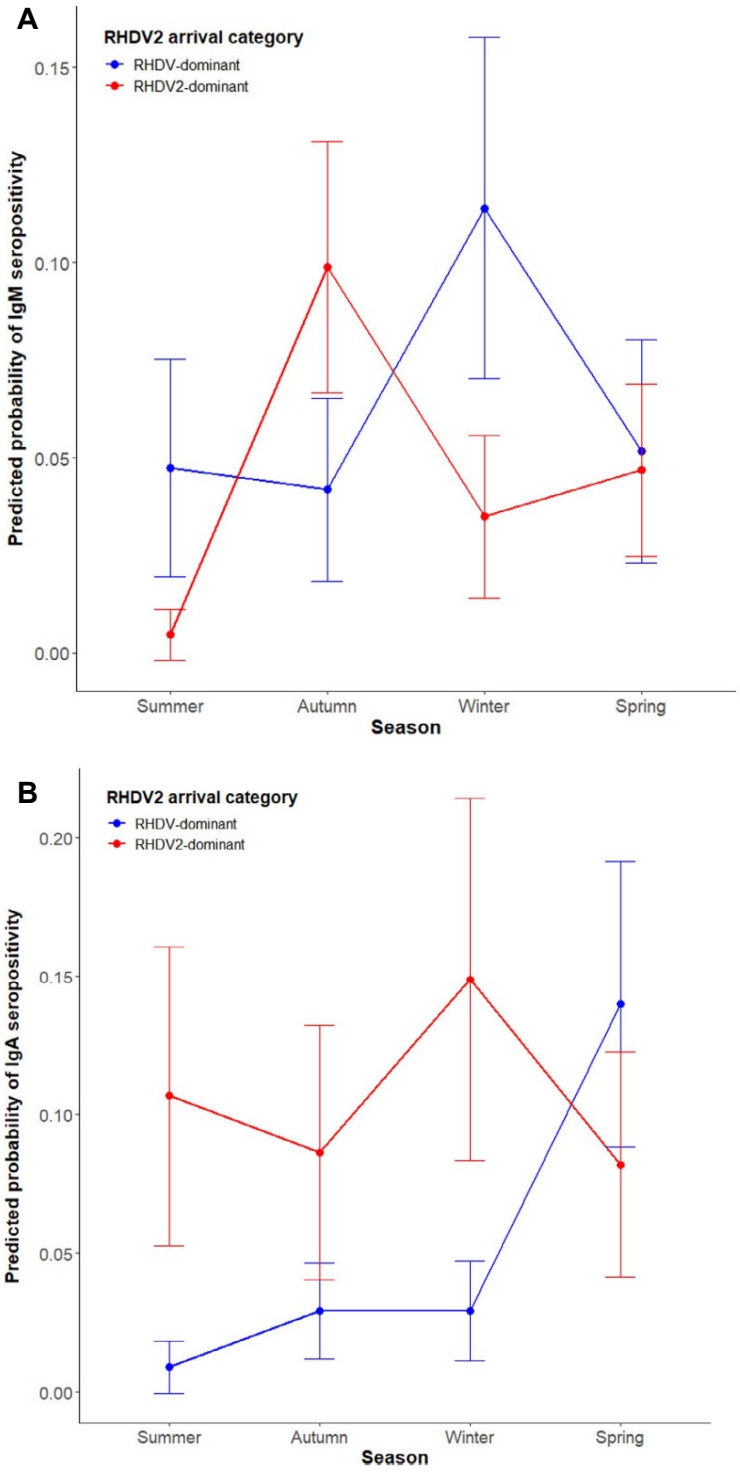


*Figure 9. Seasonal dynamics of (A) IgM and (B) IgA seropositivity within an RHDV-dominant versus RHDV2-dominant landscape. Figures demonstrate a forward shift in virus-transmission dynamics by approximately one season. Source: Taggart et al. 2022.*

Since 2018, the ongoing rabbit monitoring at six sites across Australia carried out through this project confirms that the seroprevalence of short-lived IgM and IgA RHDV2 antibodies is high and that virus transmission is frequent (Figure 10; Figure 11). IgM seroprevalence in data from 2018 onwards frequently exceeds 20% and commonly reaches 50%, and IgA seroprevalence frequently exceeds 50% and commonly reaches 75% or higher. Given that both IgM and IgA antibodies are short-lived, these high seroprevalence values and frequencies of detection confirm frequent RHDV2 transmission across the majority of the ongoing quarterly rabbit-biocontrol monitoring sites. Similar high

seroprevalence values are also observed for RHDV2 IgM and IgA antibodies at the longitudinal Turretfield study site (Figure 12), again suggesting frequent RHDV2 transmission at this site.

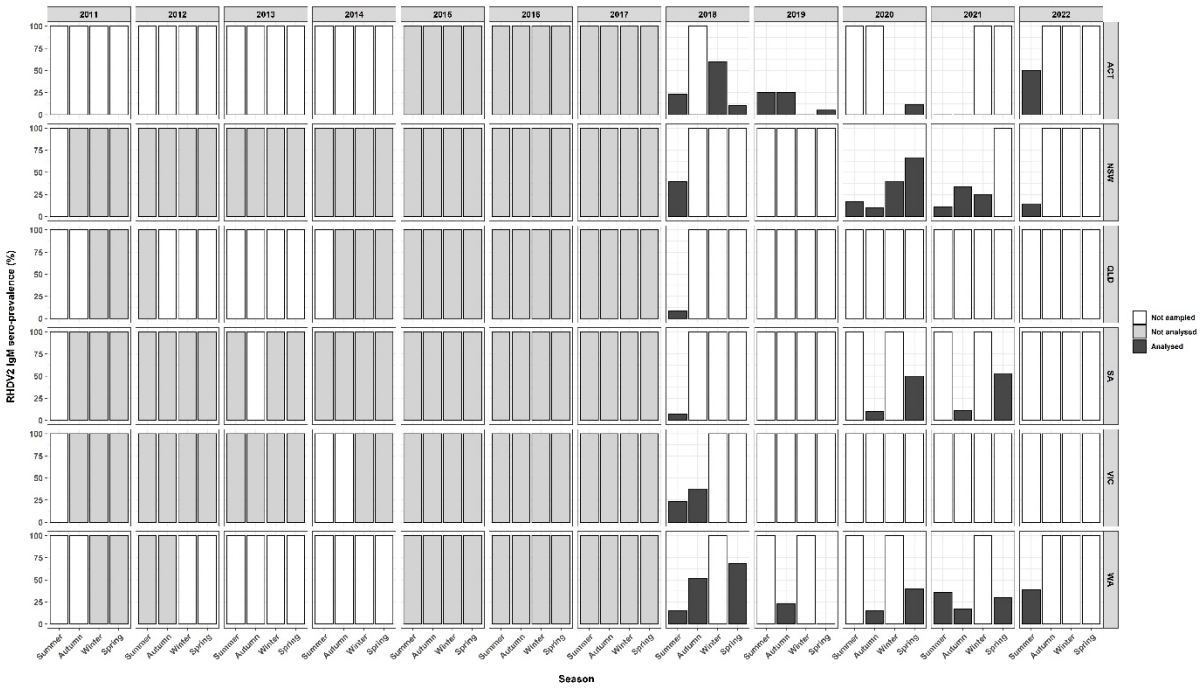


*Figure 10. RHDV2 IgM seroprevalence by state and season. Source: Kandarp Patel*

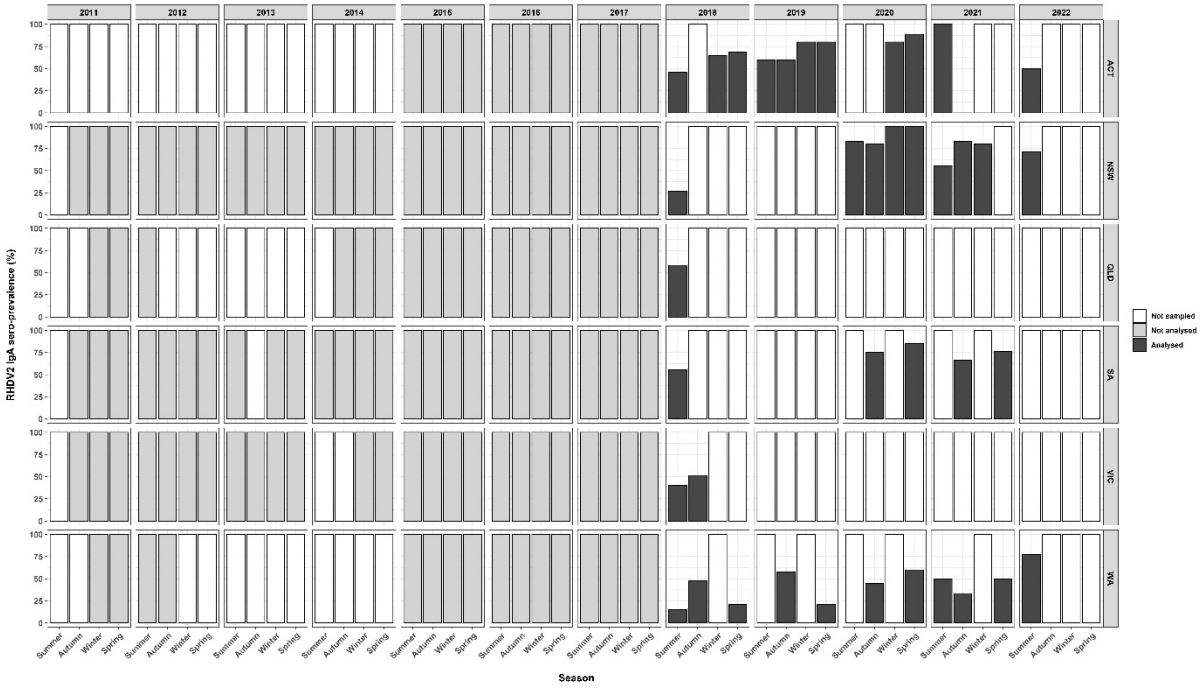


*Figure 11. RHDV2 IgA seroprevalence by state and season. Source: Kandarp Patel*

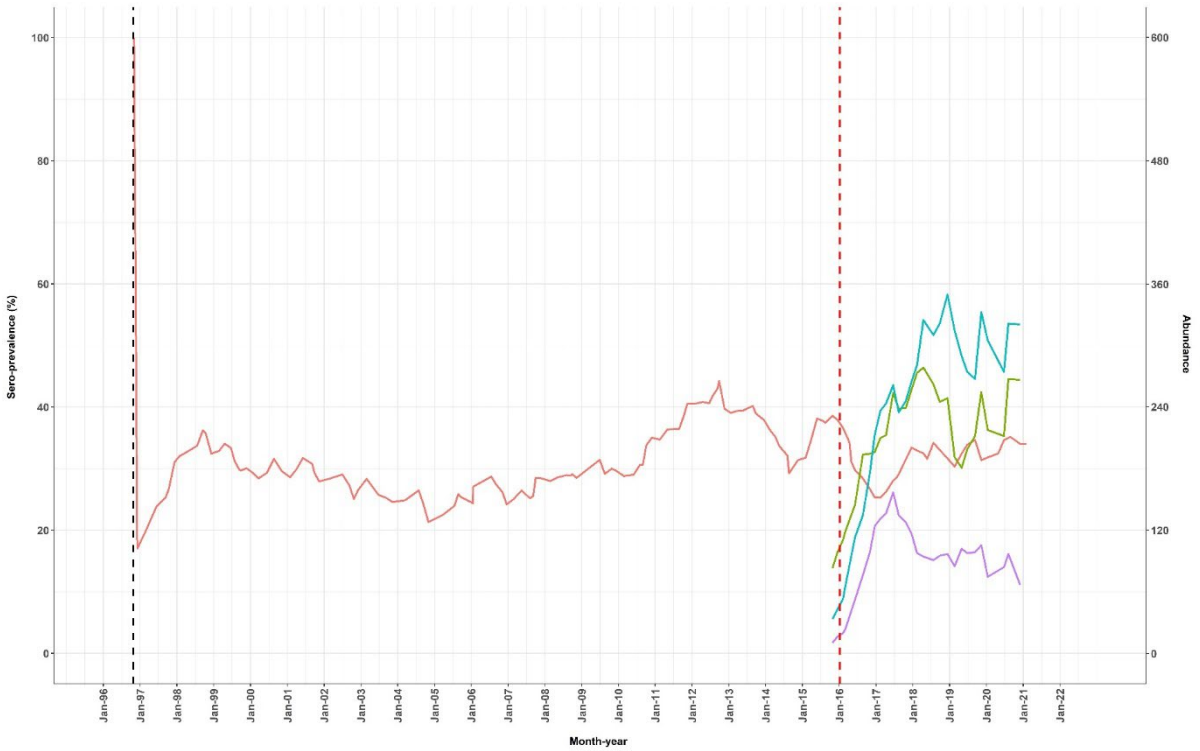


*Figure 12. Smoothed (± 3 months) seroprevalence for RHDV2 cELISA (green), IgA (blue) and IgM (violet) on the primary y-axis (left) and smoothed (± 3 months) rabbit abundance (red) at Turretfield since 1996 (as estimated using a POPAN model) on the secondary y-axis (right). The black- and red-dotted vertical lines show the release of RHDV and the arrival of RHDV2 at Turretfield, respectively. Source: Kandarp Patel.*

## COMPONENT F6: PATTERNS IN THE SUPPLY AND RELEASE OF RHDV

### *METHODS*

If RHDV2 were to be registered as an official rabbit biocontrol in Australia and supplied to land managers, its use could be expected to be like that of the currently registered RHDV product. With this in mind, patterns in the use and supply of RHDV are directly relevant to the possible registration and use of an RHDV2 product. For these reasons, Taggart et al. (2022b) obtained data on the supply and release of RHDV to land managers across the country. They then summarised all known published studies on rabbit-breeding patterns in Australia, investigated when RHDV was supplied to land managers and when it was released relative to when we would expect rabbits to be breeding and young rabbits to be present in wild populations. As young rabbits are immune to lethal RHDV infection, it is recommended that RHDV should not be released into wild-rabbit populations when young rabbits are present; doing so would be expected to introduce RHDV immunity into the population and possibly lead to rabbit populations becoming harder to control in future.

### *RESULTS*

Taggart et al. (2022b) found that half of all RHDV supply (47%) and three-quarters (74%) of reported releases Australia-wide occurred during the anticipated major rabbit-breeding seasons and when the risk of immunising young rabbits is greatest (Table 4). RHDV supply and release occurred during the anticipated major rabbit-breeding seasons in almost all Australian states.

*Table 4. Cumulative RHDV supply from government-curated records and reported releases from RabbitScan by land managers for each month, season and state. Source: Taggart et al. (2022b).*

| | Total number of RHDV supply/releases | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Summer | | Autumn | | | Winter | | | Spring | | | Summer | |
| State | Jan | Feb | Mar | Apr | May | Jun | Jul | Aug | Sep | Oct | Nov | Dec | Total |
| RHDV supply | 59 | 81 | 127 | 104 | 80 | 74 | 52 | 66 | 97 | 89 | 105 | 62 | 996 |
| Aust. Capital Territory | 1 | 4 | 5 | 3 | 3 | 0 | 0 | 2 | 3 | 1 | 0 | 0 | 22 |
| New South Wales | 11 | 33 | 50 | 44 | 24 | 26 | 17 | 24 | 28 | 24 | 43 | 22 | 346 |
| Northern Territory | 0 | 1 | 0 | 3 | 1 | 1 | 0 | 2 | 4 | 3 | 0 | 0 | 15 |
| Queensland | 4 | 10 | 6 | 10 | 13 | 11 | 7 | 19 | 18 | 19 | 10 | 7 | 134 |
| South Australia | 7 | 2 | 6 | 8 | 5 | 8 | 3 | 4 | 11 | 6 | 4 | 3 | 67 |
| Tasmania | 4 | 5 | 7 | 1 | 5 | 2 | 0 | 1 | 0 | 0 | 1 | 2 | 28 |
| Victoria | 19 | 10 | 33 | 17 | 19 | 17 | 19 | 11 | 11 | 22 | 14 | 14 | 206 |
| Western Australia | 13 | 16 | 20 | 18 | 10 | 9 | 6 | 3 | 22 | 14 | 33 | 14 | 178 |
| RHDV releases | 14 | 25 | 12 | 87 | 2 | 21 | 16 | 15 | 20 | 111 | 173 | 113 | 609 |
| New South Wales | 1 | 6 | 3 | 0 | 1 | 6 | 1 | 0 | 3 | 62 | 2 | 0 | 85 |
| Northern Territory | 0 | | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| Queensland | 0 | 3 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 0 | 0 | 7 |
| South Australia | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 9 | 1 | 3 | 1 | 15 |
| Tasmania | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Victoria | 0 | 8 | 8 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 20 |
| Western Australia | 13 | 6 | 0 | 86 | 0 | 15 | 14 | 14 | 7 | 46 | 168 | 111 | 480 |

*Note*: RHDV supply and releases data relate to the periods 1997–2021 and 2011–2021, respectively.
Abbreviation: RHDV, rabbit hemorrhagic disease virus.

## COMPONENT F7: ASSESSING BLOWFLY ANALYSIS AS AN ADDITIONAL METHOD TO STUDY LANDSCAPE-SCALE EPIDEMIOLOGY OF RHDVS

### *METHODS*

This component aimed at assessing the suitability of fly monitoring to gather additional data on the landscape-scale virus activities of the various circulating RHDVs. Carrion-feeding blowflies are known to be mechanical vectors of RHDV (Asgari, Hardy, Sinclair and Cooke 1998) and can reflect the epidemiological patterns at selected sites (Hall, Huang, Roberts and Strive 2019). For this project, a national network for regular flytrapping was established. Scientific and collaborative networks of project members were used to establish 18 sites across the country; traps, bait and return envelopes were provided to collaborators for the fortnightly trapping of flies. Fly samples were returned for testing quarterly and were analysed according to previously described methods (Hall et al. 2019).

This project component was originally intended to be a PhD project. Due to the exploratory nature of PhD projects, no contractual milestones were associated with this component. As no suitable PhD candidates could be recruited, this project aspect was later converted into a side project and some of the objectives were addressed using project resources.

### *PRELIMINARY RESULTS*

Between March 2019 and November 2021, a total of 358 fly samples from 18 sites were analysed. Fifty-five of these samples (15%) tested positive for RHDV2; no other RHDVs were detected in flies. This data was combined with the PCR-testing data from Component F1, resulting in 155 fly samples that had also had at least one rabbit sampled within 10 days either side of the fly sample and within a 50 km radius – this is referred to as ‘nearby’. The analysis of this data will involve occupancy modelling and is ongoing in collaboration with Professor Richard Duncan from the University of Canberra. We estimate results to be available and submitted for publication in late 2023, and

anticipate the findings to inform the usefulness of this method for continent-scale monitoring of disease activity, as well as recommendations on sampling frequencies.

## AN ECONOMIC ASSESSMENT OF RHDV2

*METHODS*

By mid-2021 the cumulative data from both projects converged to suggest that a registration and subsequent rollout of RHDV2 as a product was not likely to yield substantial benefits. Following advice from the CISS Rabbit Biocontrol Steering Committee, the final cumulative data was summarised in early 2022 and used to inform a cost-benefit analysis carried out by an external provider (ACRE Economics) (Hardaker 2022) based on established approaches to assess the benefits of previous biocontrol agents (Hardaker and Chudleigh 2020).

*RESULTS*

Hardaker (2022) conducted an economic cost-benefit analysis of RHDV2 registration and estimated that the expected net benefits of investment to facilitate the registration, approval and release of an RHDV2 biocide were approximately $0.69 million (present value terms, over 30 years using a five per cent discount rate). However, the best-case cost scenario – where no additional non-target species testing is required – suggested total nominal investment costs of $3.2 million over a period of five years equivalent to $2.64 million in present value terms. The discounted benefit and cost cash flows gave a net present value of −$1.95 million and a benefit-cost ratio of 0.26.

Hardaker (2022) suggested that the estimated total expected net benefits of just $0.69 million and corresponding investment criteria estimated show that the additional investment required to achieve full registration and approval of a RHDV2 biocide is unlikely to generate a positive return on investment. Further, the analysis identified several issues associated with a RHDV2 biocide that support the quantitative findings. Such issues include:

- RHDV2 would need to be listed separately to ‘rabbit calicivirus disease organisms’ under the *Biological Control Act 1984* which would necessitate a public consultation process
- evidence from existing biocide use indicates that almost three-quarters of reported biocide releases are misapplied by land managers
- early data has shown that RHDV1-K5 appears to be better able to overcome RHDV2 immunity, relative to RHDV2 overcoming RHDV immunity or RHDV2 overcoming RHDV2 immunity.

Hardaker (2022) additionally conducted an analysis of potential investment to increase and improve the use of the existing RHDV1-K5 biocide to provide a point of comparison for the RHDV2 biocide registration. The RHDV1-K5 comparison analysis had an estimated total expected net benefit of $2.17 million (present value terms) against potential costs of $0.68 million (present value terms). This gave an estimated net present value of $1.49 million and a benefit-cost ratio of approximately 3.2:1 over 30 years using a five per cent discount rate. Investment criteria were positive from 10 years from the first year of investment assumed.

Based on their results, Hardaker (2022) recommended that rabbit-biocontrol and invasive-species stakeholders continue to monitor and evaluate the wild-rabbit population and changing environment with respect to existing biocontrol agents (RHDV1 strains and endemic RHDV2), and that additional investment into increasing and improving the use of the existing RHDV1-K5 biocide may be worthwhile.

# DISCUSSION AND RECOMMENDATIONS

## WHAT WE KNOW NOW THAT WE DIDN'T IN 2017

Following its emergence in 2010, RHDV2 quickly spread across the entire globe, affecting wild and domestic lagomorph (order Lagomorpha) populations worldwide. Following its arrival into Australia, its properties were poorly understood, especially because findings from European RHDV2 isolates varied greatly and were often contradictory – rendering predictions of its impacts and potential effects of the pending RHDV-K5 release very difficult.

The five-year research program described provided the most thorough characterisation of circulating RHDV2 variant anywhere in the world by investigating its biological and immunological properties, welfare impacts, distribution and spread, ongoing evolution and adaptation, impact on Australian rabbit populations and interactions with other circulating viruses. It also supported the development of a vaccine to protect non-target owned rabbits from viral biocontrols. The resulting comprehensive cumulative dataset was used to assess the suitability of RHDV2 as an additional biocide tool, make evidence-based recommendations regarding the worth of proceeding with registering RHDV2, and develop recommendations on how to proceed with the optimisation of viral biocontrols.

## IMPLICATIONS AND OPPORTUNITIES

On balance, the cumulative results suggest that the registration of RHDV2 would be unlikely to result in substantial gains.

One of the few findings that could lend support to a potential registration was that the Australian RHDV2 is highly virulent in susceptible adult and young rabbits, with welfare impacts comparable to previously registered RHDV strains.

However, multiple project outputs provided strong arguments against a registration at this point in time. A high frequency of RHDV2 transmission in wild populations indicated by increased seroprevalence of short-lived (IgM/IgA) antibodies, and frequent detection of RHDV2 positive cases in dead rabbits indicate that RHDV2 is currently active very frequently and gets into susceptible rabbit populations very quickly. The resulting high levels of average seroprevalence (about 60% on average) would make it very difficult to find a window of opportunity to release RHDV2 and achieve a meaningful local population knockdown. For maximum efficiency from RHDV release, prior fast-turnaround serological testing of wild-rabbit populations should ideally be conducted. However, due to the time, effort, capability and infrastructure required to sample rabbits and conduct such testing, it is rarely done.

Our data also shows that passive maternal immunity to RHDV2 protects from lethal disease, but not infection, in a dose-dependent manner. While there is no innate age-related immunity, based on these findings a year-round release of RHDV2 – including during the breeding season – cannot be recommended. By analysing data on the supply of registered and available RHDV products to land managers, we found that these are regularly used inappropriately and released at the wrong time of the year: potentially up to 75% of the time. This is of significant concern and is likely contributing to making rabbits harder to control in future. The same pattern of inappropriate use would be expected for an RHDV2 registered product.

The potential registration of an ineffective new biocide product also bears a reputational risk. This is especially the case given that K5, the last rabbit biocontrol given to land managers, also received very mixed reviews and achieved minimal rabbit knockdown in many cases (Cox et al. 2019).

The combined findings were used for an economic impact assessment by ACRE Economics that used modified methodologies from previous impact assessments of RHDV-K5 and RHDV2. The results of this independent analysis confirm the case against proceeding with RHDV2 product registration, suggesting a net present value of −$1.95 million and a benefit-cost ratio of 0.26.

Ongoing epidemiological monitoring shows that RHDV2 continues to change and evolve, and changes in epidemiological patterns may lead to a different scenario where there could potentially be benefits in a registered product. In spite of this, there are likely few (if any) situations where accessing RHDV2 for release immediately would drastically change the outcomes of rabbit management and rabbit impacts for land managers, compared with waiting an additional two or three years.

Based on these combined recommendations, the CISS Rabbit Biocontrol Steering Committee made the recommendation not to proceed with the registration process, but to write up the registration package as far as possible with the available data and shelve it for potential future reactivation. This recommendation was tabled and received endorsement at the Environment and Invasives Committee in August 2022.

While the cumulative data did not support the registration of RHDV2, the data suggest that the registered and available RHDV-K5 may now be a better biocide tool than in 2017 when it was released nationwide, and when wild populations had widespread and completely protective RHDV (-Czech) immunity. In our studies, RHDV (including K5) was better able to overcome RHDV2 immunity. In laboratory trials, all rabbits that were vaccinated against RHDV2 were killed by RHDV infection (12/12 rabbits). RHDV (including K5) infections of rabbits with natural immunity to RHDV2 resulted in 34% case fatality rates on average. In field trials, nine to thirteen per cent of rabbits with natural (recent) RHDV2 immunity were killed by RHDV infection (Czech or K5).

With RHDV2 now the dominant virus in the landscape and the resulting RHDV2 population immunity, RHDV-K5 would have greater efficacy by affecting not just susceptible seronegative rabbits but also a proportion of RHDV2-immune animals. This presents a new opportunity, and future studies are warranted to optimise the use of RHDV-K5 in light of these findings to maximise benefits from targeted biocontrol applications.

## THE WAY FORWARD: MONITORING AND EVALUATING THE EVOLVING EPIDEMIOLOGY

While RHDV2 registration and rollout was not recommended, combined project outputs have led to recommendations about how to proceed with approaches aimed at optimising rabbit biocontrol. The ongoing monitoring efforts and serological surveys show that RHDV epidemiology remains dynamic and is evolving. The parallel molecular diagnostic work continues to provide an excellent public-engagement tool and yields valuable data on strain composition, genetic variability and the emergence of new recombinants. The data illustrates that the virus is continuing to co-evolve and adapt to the Australian conditions. RHDV-K5 has shown potential to be a better biocide today than seven years ago.

This is supported by the independent economic analysis (Hardaker 2022) that included an analysis of potential investment to increase and improve the use of the existing RHDV1-K5 biocide, and estimated a net present value of $1.49 million and a benefit-cost ratio of approximately 3.2:1 over 30 years. Based on their results, Hardaker (2022) recommended that rabbit-biocontrol and invasive-species stakeholders continue to monitor and evaluate the wild-rabbit population and changing environment with respect to existing biocontrol agents (RHDV1 strains and endemic RHDV2), and that additional investment into increasing and improving the use of the existing RHDV1-K5 biocide may be worthwhile.

The combined findings led to the development of further project concepts proposing:

- a continuation of monitoring and evaluating the naturally circulating viruses. This includes all circulating RHDVs and myxoma virus, which has been implicated in reduced population survival when followed by an RHDV outbreak (Barnett et al. 2018)
- identification and use of possible windows of opportunity for more tailored applications

- development of smarter and more efficient monitoring methods for both rabbits and viruses
- development of better strategies for increasing the integration of existing biocontrols with conventional tools and demonstration of the long-term benefits of such integration to encourage uptake.

A key component of monitoring and evaluating naturally circulating viruses going forward also includes building and strengthening the diagnostic capability in Australia (serological and molecular) and investing in experimental work to optimise the available K5 formulation to achieve maximum possible impact through targeted virus releases.

In addition, the finding that that a large proportion of RHDV is either supplied or released during the major anticipated rabbit-breeding seasons – when the risk of immunising young rabbits is greatest – suggests that a large majority of RHDV releases are likely inappropriate and unseasonal. This potentially does significant damage and likely makes rabbit populations harder to control in the future. A critical component of any optimisation of RHDV-K5 as a biocontrol tool must be ensuring no unintended negative consequences, by improving the education about release times or restricting periods where biocontrol products can be supplied/released. This must be done in conjunction with education on best practice methods of applying integrated rabbit control.

Lastly, critical to all of this and optimising the use of existing biocontrols going forward, we must better understand what actually happens following the release of any biocontrol. Despite RHDV having now been released for over 20 years, understanding of what happens after release is very poor. Such understanding includes asking and answering questions such as: Which rabbits are killed by the released virus? Does it kill only those rabbits that consume the virus-treated bait? Does it spread within the release warren to animals beyond those directly consuming the virus-treated bait? Does it spread to neighbouring warrens or further? How far does the released virus travel and, consequently, how should virus releases be spaced in the landscape to achieve effective coverage at regional or landscape scales? Answering such questions will require well-planned and detailed field experiments, but will vastly improve how the currently registered virus is used to achieve optimal rabbit control.

The proposals are in line with the updated 20-year biocontrol research and development pipeline strategy in offering immediate options going forward. The strategy also includes additional medium- to long-term recommendations, including identification of novel biocontrols, accelerated selection for improved RHDV variants and investment into long-term genetic biocontrol strategies.

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