P.PSH.1059 - Final report

Strengthening the viral rabbit biocontrol pipeline for sustainable long-term rabbit control

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Abstract

This project addressed a critical gap in Australia's rabbit biocontrol research by developing medium- to long-term strategies to complement current control methods. It has led to scientific advancements in line with the long-term rabbit biocontrol research and development strategy towards a pipeline of innovative rabbit biocontrol tools ready to deploy in 10- to 15-year intervals.

A major breakthrough was the establishment of the world's first robust cell culture system for the *Rabbit haemorrhagic disease virus (RHDV)*, providing a foundation for developing new virus variants for future rabbit control. Whole-genome sequencing of over 300 rabbits facilitated the identification of targets for potential genetic control strategies, while metagenomic analysis identified the *Hepatitis E virus* in Australian rabbits and novel hepaciviruses in North American lagomorphs. Additionally, the specificity of RHDV2 for lagomorphs was confirmed, ensuring its safety for non-target species.

The economic feasibility of releasing a new RHDV variant developed through the RHD-Accelerator approach, as part of the long-term rabbit biocontrol pipeline, was evaluated. Economic modelling showed a high return on investment ranging from 97:1 to 6:1 depending on the properties of the putative new variants, making a compelling case for continued investment in this technology.

This project resulted in a total of seven publications in peer-reviewed international journals to date, with an additional two submitted and another and a third close to submission. The project outputs lay the groundwork for future innovative and sustainable rabbit management solutions that will benefit both Australian agriculture and the broader environment.

Executive summary

Background

The objective of this project was to close a large gap in the rabbit biocontrol research portfolio pursued in Australia between 2017 and 2022. Supported through the Centre for Invasive Species Solutions (CISS), rabbit biocontrol work research during that time focussed on the short-term optimisation of existing biocontrols and some stakeholder engagement work on future technologies such as long-term genetic biocontrol strategies. As no medium-term approaches were being pursued with a research and development timeframe of 10–15 years, this project aimed to fill this gap. It was mainly focussed on medium-term approaches for novel biocontrol tools, but also explored future technologies such as gene drives, thereby contributing to a more comprehensive rabbit biocontrol research pipeline for the sustainable landscape-scale suppression of rabbit impacts in Australia.

The beneficiaries of this research are primary producers impacted by rabbits. The goal of this research was to develop future biocontrol tools that keep rabbit populations at manageable levels, leading to improved pasture quality, reduced land degradation and greater resilience against drought. In addition, effective new rabbit biocontrol tools will lead to substantial and sustained environmental benefits, including on-farm biodiversity.

The findings from this research will serve as a foundation for future research, including the development of the next generation of biological controls for rabbits and potential advancements in the genetic engineering of rabbits for long-term population management.

Objectives

The aims of this project were to:

- Develop a rabbit organoid platform to support in vitro replication of *Rabbit haemorrhagic disease virus* (RHDV), continuation of the RHD-Accelerator platform technology aimed at selecting immune escape variants for subsequent virus releases. **Achieved.**
- Investigate unexplained deaths in lagomorphs in Australia and overseas to potentially identify future biocontrol agents or biocides. **Achieved.**
- Undertake work to demonstrate any trace level virus RNA that may be present in non-target scavengers of rabbit to support RHDV2 registration by the APVMA. **Achieved.**
- Commission a modelling study investigating the theoretical feasibility for genetic biocontrol options for rabbits in Australia. **Achieved.**
- Estimate the likely cost-benefit of implementing the proposed long-term rabbit biocontrol pipeline. **Achieved.**

Methodology

The development and optimisation of advanced culture systems for RHDV was achieved by adapting and optimising advanced organoid cell culture techniques described in the scientific literature for rabbit livers.

Next generation sequencing technologies (metatranscriptomics) was used for pathogen discovery in rabbits and other lagomorphs using animals that were found dead but did not die from known biocontrols in Australia and overseas.

Standard virological diagnostic tools were applied to test for the presence of RHDV and/or antibodies to RHDV in feral Australian scavengers (cats, dogs, foxes and pigs)

Whole genome sequencing was carried out for over 300 rabbits. The data enables the identification of suitable gene targets for future Australia-specific genetic biocontrol strategies. Furthermore, the data will provide a baseline for modelling the spread of a putative gene drive in Australian rabbits.

The continuation of the RHD-Accelerator platform technology was selected for an in-depth economic assessment on the impacts of implementing this pipeline, in line with Australian Government best-practice guidelines for cost-benefit analyses.

Results/key findings

This project successfully established the world's first cell culture system capable of supporting RHDV replication, paving the way for future work utilising natural selection processes to produce new biocontrol agents. The project also identified the Hepatitis E virus in Australian rabbits and discovered novel hepaciviruses in North American lagomorphs, highlighting the effectiveness of the methodology for this type of bioprospecting. However, none of the viruses identified was deemed a strong candidate for biocontrol. The study also confirmed RHDV2's host specificity, demonstrating that the virus does not infect non-lagomorph species. Furthermore, population-level genomic analysis provided the baseline enabling the identification of potential gene drive targets and laying the groundwork for future genetic biocontrol strategies. Finally, economic modelling demonstrated that investing in new RHDV-based biocontrol agents produced through the RHD-Accelerator platform would likely result in high returns on investment, with an estimated Net Present Value of AUD $1.43 billion over 40 years. Although not without risks, the possibility for repeat applications of the RHD-Accelerator approach and resulting repeated, compounded impact presents additional opportunities to gradually reduce rabbit populations to levels not seen since the introduction of the first rabbit calicivirus in 1996.

Benefits to industry

This project supports the grazing industries by advancing long-term rabbit biocontrol strategies, reducing the impact of this major pest on Australian grazing lands. The development of a culture system for RHDV and the identification of potential gene drive targets pave the way for more effective and sustainable rabbit population management. Additionally, the economic assessment highlights the value of investing in the development of medium- to long-term new biocontrol tools for the industry.

Future research and recommendations

The project provides a strong business case to continue RHD-Accelerator research and leverage the organoid platform to accelerate the natural selection of new virus variants and produce new RHDV-based rabbit biocontrol candidates for potential future registration and release in Australia. A continued watching brief should be kept on any new emerging rabbit pathogens globally, including any new emerging RHDV variants. Strengthening international collaborations will be critical for identifying future potential biocontrol agents outside Australia should these emerge. For longer-term technology-driven biocontrol strategies such as gene drives, preliminary genetic modelling indicates that is approach is theoretically feasible and warrants further investigation. This should include commencing the technical development of gene drives in rabbits, as well as additional modelling that combines genetic, biological and ecological aspects to enhance predictive accuracy of gene drives for rabbit control. For all novel control technologies (viral and genetic), early and

ongoing engagement with regulators and policy makers as well as public engagement will be critical to ensure a transparent and responsible translation of these innovative tools for sustainable management of rabbit impacts across Australia.

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1. Accelerator (rabbit organoids)

1.1 Background – Accelerator

The first R*abbit haemorrhagic disease virus* (RHDV1) was detected in the 1980s in China (S. J. Liu et al., 1984) and has since spread worldwide. The virus causes high mortality rates in European rabbits and was successfully used as a biocontrol agent in Australia to supress feral rabbit populations. In 2010, a related virus (hereafter referred to as RHDV2) emerged in France (Le Gall-Reculé et al., 2011). Like its predecessor, it spread rapidly worldwide. However, in contrast to RHDV1, RHDV2 has an extended host range killing hares and other lagomorphs. Although RHDVs have been known for several decades and have a relatively small genome (7.5 kb) encoding just eight proteins, there are still significant gaps in our understanding how it infects and replicates in cells. This is largely due to the lack of a robust cell culture system. Until recently, all studies involving virus propagation had to be carried out in live rabbits, which is both cost- and labour intensive. The advancement of organoid technology, i.e. 3D cell culture systems that contain multiple cell types and mimic organs in composition and function, has created new opportunities for growing RHDV in the laboratory. Such systems could be invaluable for virus characterisation, propagation, titration, vaccine development and the generation of new virus variants for biocontrol. Their availability would significantly reduce research and development costs and animal use.

To decrease Australia's reliance on the serendipitous emergence of new rabbit diseases to feed the rabbit biocontrol pipeline, a concept termed 'RHD-Accelerator' was developed as part of long-term the rabbit biocontrol pipeline strategy (Centre for Invasive Species Solutions, 2024). It aims at accelerating and guiding virus evolution to select for novel antigenic variants or serotypes through the use of neutralising antibodies. A previous project co-funded by MLA explored this approach by passaging virus in passively immunised rabbits and delivered proof of concept, however no new variant was selected as the need to carry out every experiment in rabbits was too resource intensive and time consuming (Hall et al., 2017).

An organoid-based cell culture system could facilitate the development of an animal-free RHD-Accelerator platform and allow for a faster selection process at greatly reduced costs. The use of organoid systems for virus replication had already shown great promise for the cultivation of human noroviruses in intestinal organoids, a significant breakthrough for another difficult-to-propagate member of the Caliciviridae family (Costantini et al., 2018). This project component aims at developing this organoid system to pave the way for resuming the accelerator approach of selection possible future RHDV-based biocontrol agents to future proof Australia's rabbit biocontrol pipeline.

1.2 Objectives – Accelerator

The aim of this project was to transfer intestinal organoids technology from a globally leading laboratory at the Baylor College of Medicine, (Houston, Texas, USA) and use these learnings to initially establish rabbit intestinal organoids to demonstrate replication of the benign rabbit calicivirus (RCV). The technology was then to be adapted to liver organoids to facilitate the infection and replication of RHDV1 and RHDV2 and to commence the passaging and selection of RHDV1 and RHDV2 with and without antibody selection. The approach used was:

- Method transfer to establish rabbit intestinal organoids
- Method adaptation to establish rabbit liver organoids

- Demonstrate RHDV1 and RHDV2 replication and passaging in organoid-derived cell cultures
- Commence passaging of RHDV1 and RHDV2 in the presence of neutralising antibodies
- Publication of results

All objectives of this project component have been met successfully.

1.3 Methodology – Accelerator

Technology for the generation of human and mouse intestinal organoids was transferred from the Estes lab at the Baylor College of Medicine to the Team at CSIRO. This initial work focussed on studying the benign rabbit calicivirus RCV. To characterise intestinal organoids, we employed immunofluorescence with cell-type specific markers and RT-qPCR gene expression analysis. For infection studies, 3D organoid cell cultures were transformed into monolayer cultures.

Building on the expertise gained from establishing intestinal organoids, we established rabbit liver organoids and evaluated their ability to support the replication of pathogenic rabbit caliciviruses. We produced organoids using New Zealand white rabbits euthanised by cervical dislocation or anaesthetic overdose, as well as wild shot rabbits collected from population management programs and wild rabbits trapped and euthanised by cervical dislocation. In addition to rabbit liver organoids, we established organoids from other species, opportunistically sampled from wild or feral animals (hare, cat, mouse, and fox), to assess species specificity. After a breakdown in cryopreservation storage facilities that resulted in the loss of previously established liver organoids, new tissue samples were collected to rebuild a biobank of liver organoids. The cell composition of selected liver organoids was further analysed using single-cell RNA sequencing.

Infection of RHDV1 and RHDV2 in rabbit and hare liver organoids was confirmed by RT-qPCR quantification of viral genomes and immunofluorescence detection of viral proteins. To confirm genuine replication, we included heat-inactivated virus inocula and mock-infected controls. Once infection was established in the organoid system, we continued virus passaging by using supernatant collected 48 hours post-infection to inoculate new monolayer cultures. To mitigate the effects of interferons released during infection and increase virus replication, we added Ruxolitinib (Rux), an inhibitor of the innate immune response 24hours before infection and maintained it for 72 hours post-infection. In addition, we screened a panel of interferon inhibitors to identify potential alternatives to Rux with similar or enhanced effects.

We reestablished collaboration with the International Lagovirus Reference Laboratory in Italy to obtain additional monoclonal antibodies (mAbs) against RHDV2 for selection. Passaging of both RHDV1 and RHDV2 has commenced using mAbs. The experimental setup involved infecting sub-confluent monolayer cultures with the viral inoculum and growing the virus in the presence of different concentrations of these antibodies. Viral concentrations were then quantified via qRT-PCR after 72 hours to determine the mAb concentration that still permitted replication. We also tested concentrations of neutralising polyclonal sera against RHDV2 for future passaging experiments.

For the purpose of generating additional mAbs against RHDV2, we generated RHDV2 virus-like particles (VLPs) using a baculovirus expression system to produce additional mAbs against RHDV2, in collaboration with CSIRO's Manufacturing Business Unit and the custom-antibody services of the Walter and Eliza Hall Institute (Melbourne, Australia). These antibodies were characterised using enzyme-linked immunosorbent assays, immunofluorescence assays, and Western blots. The resulting panel of additional mAbs will be used in future selection experiments.

Additionally, we developed a new monoclonal antibody targeting the RHDV2 RNA-dependent RNA polymerase. While this mAb cannot be used for virus variant selection, it is a valuable tool to study virus replication within the cells, as staining for a non-structural protein (rather than the structural capsid protein) provides a more robust demonstration of active viral replication.

1.4 Results – Accelerator

1.4.1 Establishment of rabbit intestinal organoids

Organoids were generated from both wild and domestic rabbit tissues. We successfully propagated organoids from a variety of rabbit small intestinal tissues, including the duodenum, jejunum and ileum, and we were able to culture organoids for at least 17 passages without any notable changes in morphology or growth rate. We were also able to successfully freeze and revive frozen organoids. Our newly established rabbit organoid cultures reproduced many characteristics of the parental tissue. For example, we detected (1) the presence of multiple differentiated cell types such as enterocytes, enteroendocrine cells, goblet cells and Paneth cells, (2) brush borders on enterocytes, (3) the production of mucin by goblet cells and (4) the synthesis of lysozyme by Paneth cells. The same cell types, except for Paneth cells, were also detected in differentiated monolayer cultures derived from spheroids. Our observations are generally consistent with previous reports on intestinal organoids of other species; however, we observed some notable differences. For example, rabbit small intestinal organoids spontaneously differentiated when subjected to mechanical shearing. We also found that the continuous addition of growth factor inhibitors was necessary for propagating rabbit intestinal spheroids. With these modifications, we have now established robust protocols for the generation of rabbit intestinal organoids. The methods (including experimental designs, measurements and statistical analyses) to establish those intestinal organoids have been published in *Scientific Reports* (Kardia et al., 2021). As a result of this publication, we have had other research groups inquiring about rabbit intestinal organoids for their (non-RHDV) research, demonstrating the broad applications and transferability of this technology.

We then conducted RCV infection experiments using these newly developed rabbit intestinal organoids. Organoids were inoculated, either in 3D format or as monolayers, with high doses of RCV-A1 and virus replication measured through RT-qPCR and immunofluorescence. Although we conducted several experiments in which we increased the inoculum dose, inoculated cells in the presence of glycochenodeoxycholic acid, a bile acid necessary for the cultivation of some human norovirus strains (Murakami et al., 2020), extended the inoculation period from 24 hours to 4 days. infected monolayers grown in transwell cell culture plates to selectively expose the basolateral surface in polarised epithelial cells, and scratched monolayers before inoculation to expose tight junctions, we were not able to detect any evidence of RCV replication. Importantly, the inability of RCV to replicate in this system suggests that enterocytes, enteroendocrine cells, goblet cells and Paneth cells are not target cells of RCV infection. Our results suggest that, unlike human norovirus, RCV-A1 does not productively infect enterocytes. This is an important finding to guide future work.

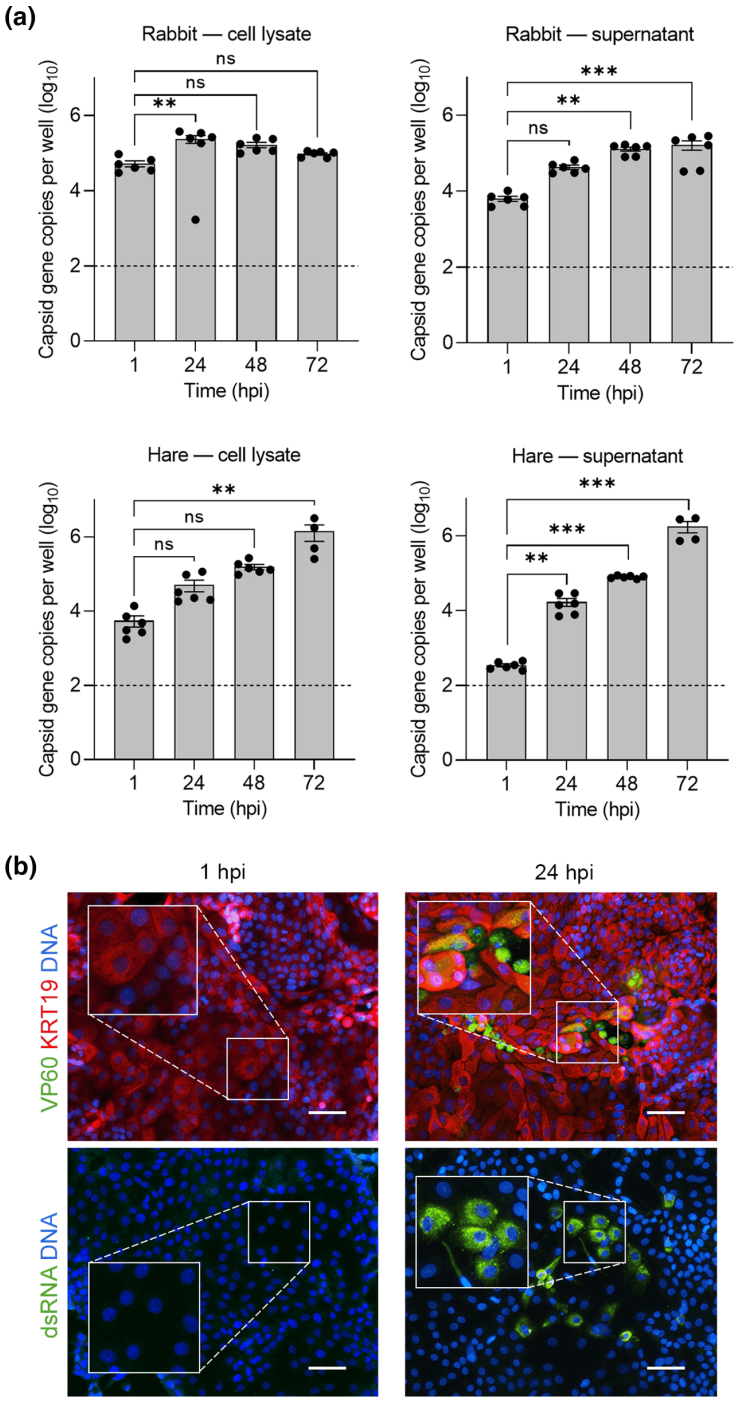
1.4.2 Rabbit liver organoids support RHDV replication

Based on experience from intestinal organoids, rabbit liver organoids were generated from liver samples collected from domestic and wild rabbits and were characterised by RT-qPCR gene expression analyses and by immunofluorescence. We detected the presence of major liver cell types,

such as hepatocytes and cholangiocytes (bile duct epithelial cells). Routine culture conditions were established and are being further optimised. Liver tissues collected from wild rabbits resulted in organoids that remained healthy over multiple passages. Organoids isolated from multiple rabbits have been cryopreserved for ongoing and future research.

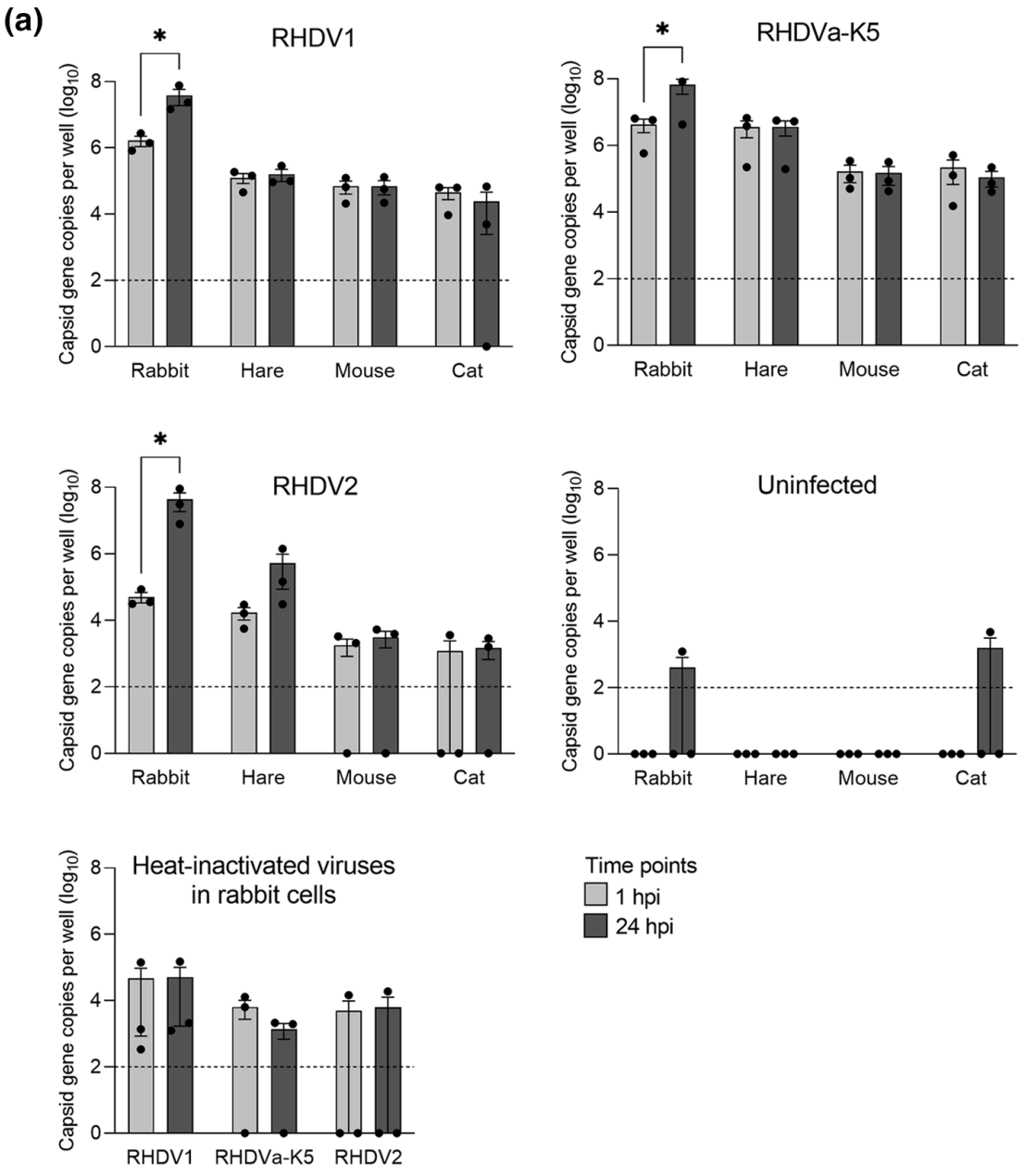
We then analysed the RHDV2 infection kinetics in rabbit and hare liver organoids (Fig. 1.1). Over a 72h period, we observed a constant level of RHDV2 RNA in rabbit organoids and a constant increase in virus titre in liver organoids derived from hare. We used immunofluorescence to demonstrate the accumulation of double-stranded RNA (dsRNA, an indicator of active virus replication) and virus capsid protein (VP60) in infected cells. The establishment of rabbit and hare liver organoids that support RHDV replication have been published in the *Journal of General Virology* (Kardia et al., 2023).

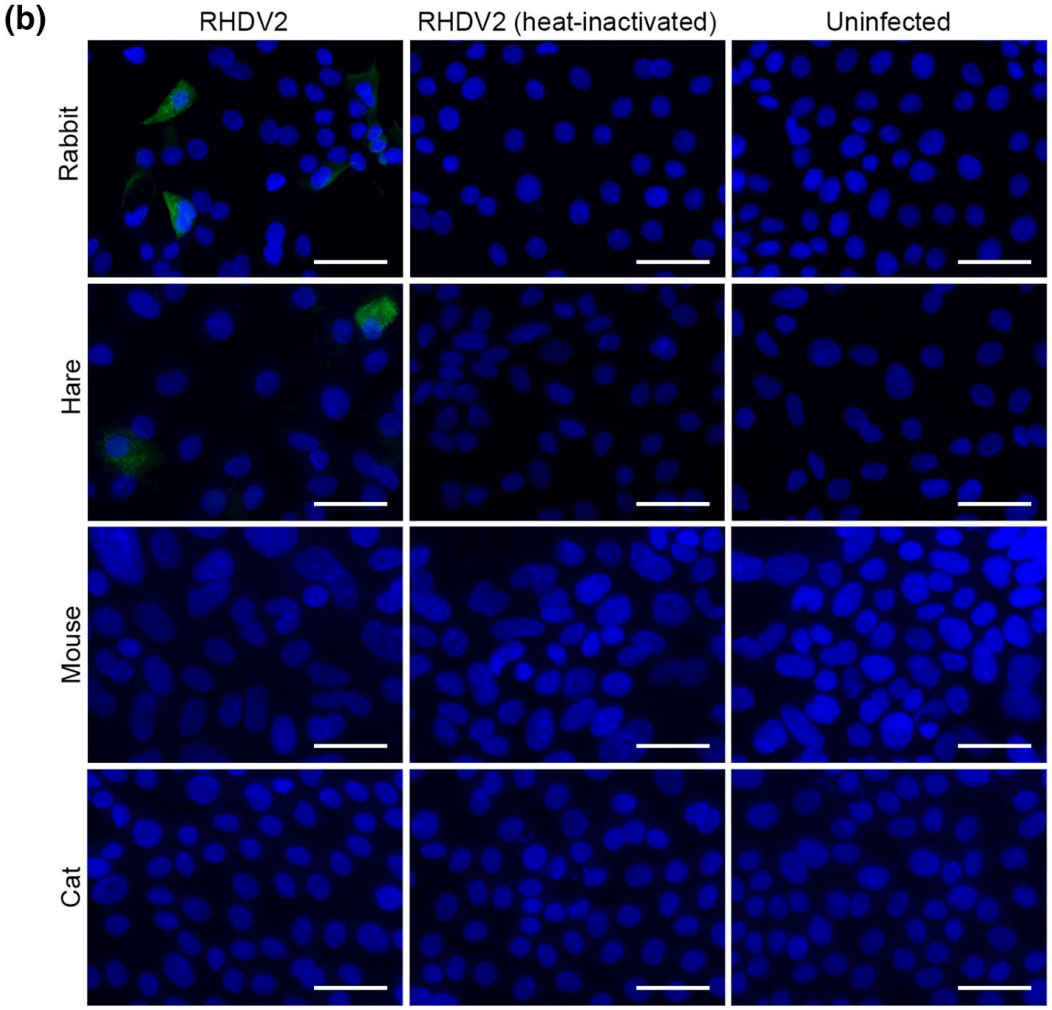
Figure 1.1: RNA replication and accumulation of viral protein in RHDV2-infected rabbit hepatobiliary organoid-derived monolayer cultures. Monolayer cultures derived from rabbit hepatobiliary organoids were inoculated with 100 RID_{50} of RHDV2 and harvested at 1, 24, 48 and 72 hpi (virus RNA quantification) or at 1 and 24 hpi (viral protein immunostaining). (a) Viral RNA quantification using RT-qPCR performed separately for cell lysates and culture supernatants. Individual data points represent at least two biological replicates with two technical replicates each. Columns and error bars represent the mean values for each time point and the standard error of the mean, respectively. The limit of quantification of the assay is shown by a dashed horizontal line. Significance is denoted by asterisks (*P≤0.05, **P≤0.01, ***P≤0.001; ns: not significant) and calculated using ANOVA followed by Tukey's HSD test, with a P-value cut-off of 0.05. (b) Immunofluorescence analysis of viral protein expression and the accumulation of dsRNA. Monolayer cultures grown in chamber slides were inoculated as described above and immunostained with mAbs directed against the lagovirus capsid protein (VP60; green in the upper panels), cytokeratin-19 (KRT19; red in all panels) or dsRNA (green in the lower panels); DNA was stained with DAPI (blue in all panels). Bars, 100 µm.



Based on protocols for the establishment of rabbit liver organoids, we also generated liver organoids from other species (cat, fox, mouse and hare) to explore if liver organoids can be used to assess species specificity in cell culture. Our results demonstrate that this is indeed the case. We found that rabbit liver organoids supported the replication of RHDV1, RHDVa-K5, and RHDV2 (Fig. 1.2). In contrast, hare cells only supported the replication of RHDV2, and cat and mouse organoids did not support any calicivirus replication, which accurately reflects the natural host ranges of these viruses (Fig. 1.2). Replication was conclusively demonstrated using a combination of an increase in viral RNA levels over time (Fig. 1.2a) and the expression of the non-structural viral RdRp as detected through immunofluorescence (Fig. 1.2b).

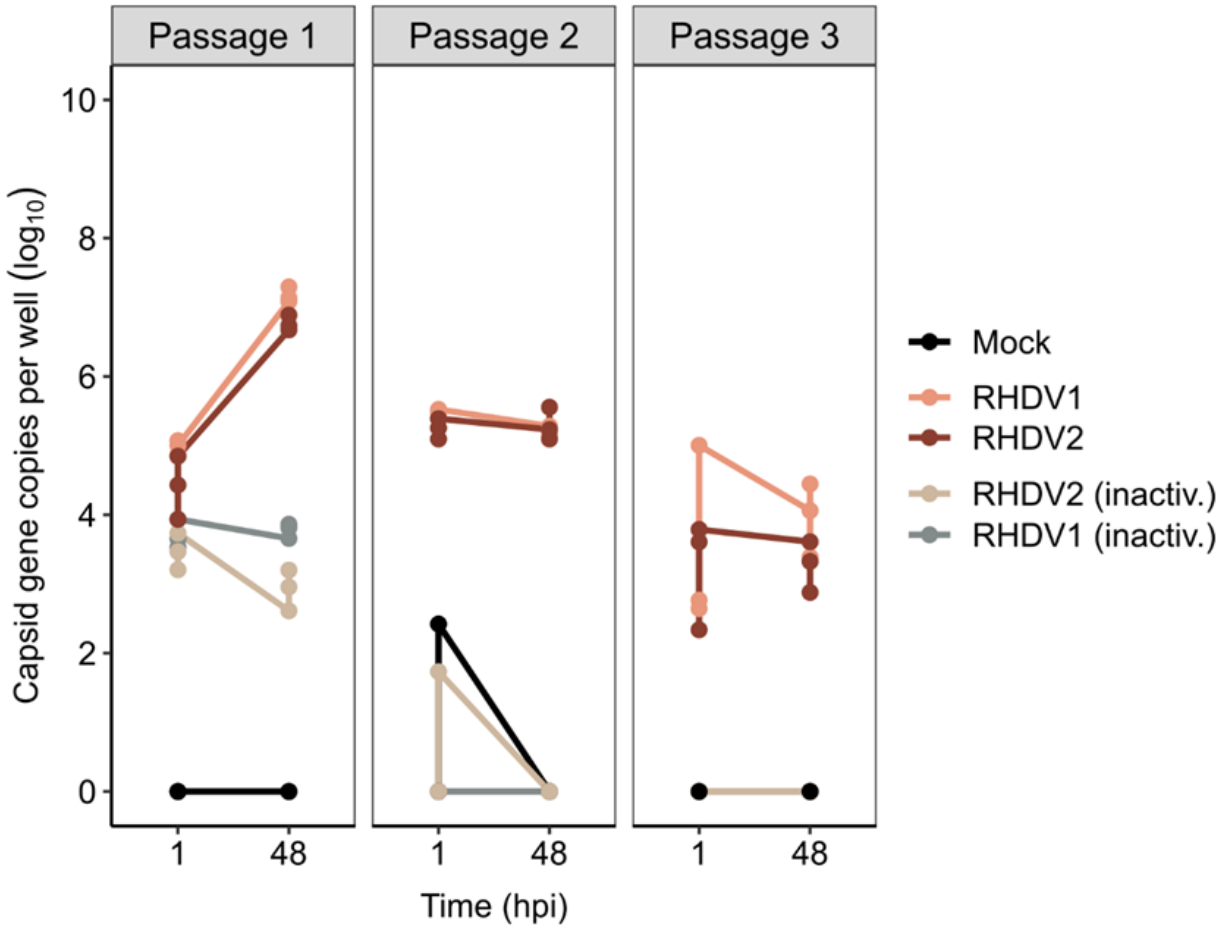
Figure 1.2: Lagovirus infection of hepatic organoids derived from susceptible and non-susceptible species. Monolayer cultures derived from rabbit, hare, mouse and cat liver organoids were inoculated with 100 '50% rabbit infectious doses' (RID_{50}) of either RHDV1, RHDVa-K5 (K5) or RHDV2. Heat-inactivated inocula and uninfected monolayers were run as controls. Monolayers were analysed at 1 and 24 hours post inoculation (hpi). (a) Viral RNA was quantified for each treatment at 1 and 24 hpi using RT-qPCR. The mean of three replicate wells is shown as a bar and the individual data points are shown at each time point (closed circles). The mean log10 values in capsid gene copies per well over 24 hours is given for each treatment. Error bars represent the standard error of the mean. (b) Monolayer cultures grown in chamber slides were inoculated with RHDV2 as described above and stained at 1 (not shown) or 24 hpi with antibodies directed against the viral RNA-dependent RNA polymerase (RdRp, green stain). Cell nuclei are stained blue. Scale bars = 50 µm.





Although the monolayer cultures were able to support the replication of RHDV1 and RHDV2, consecutive passaging of viruses was not achieved in these early experiments. The quantity of virus capsid genome, as an indicator of viral replication, was reduced to undetectable levels as soon as the third passage (Fig. 1.3).

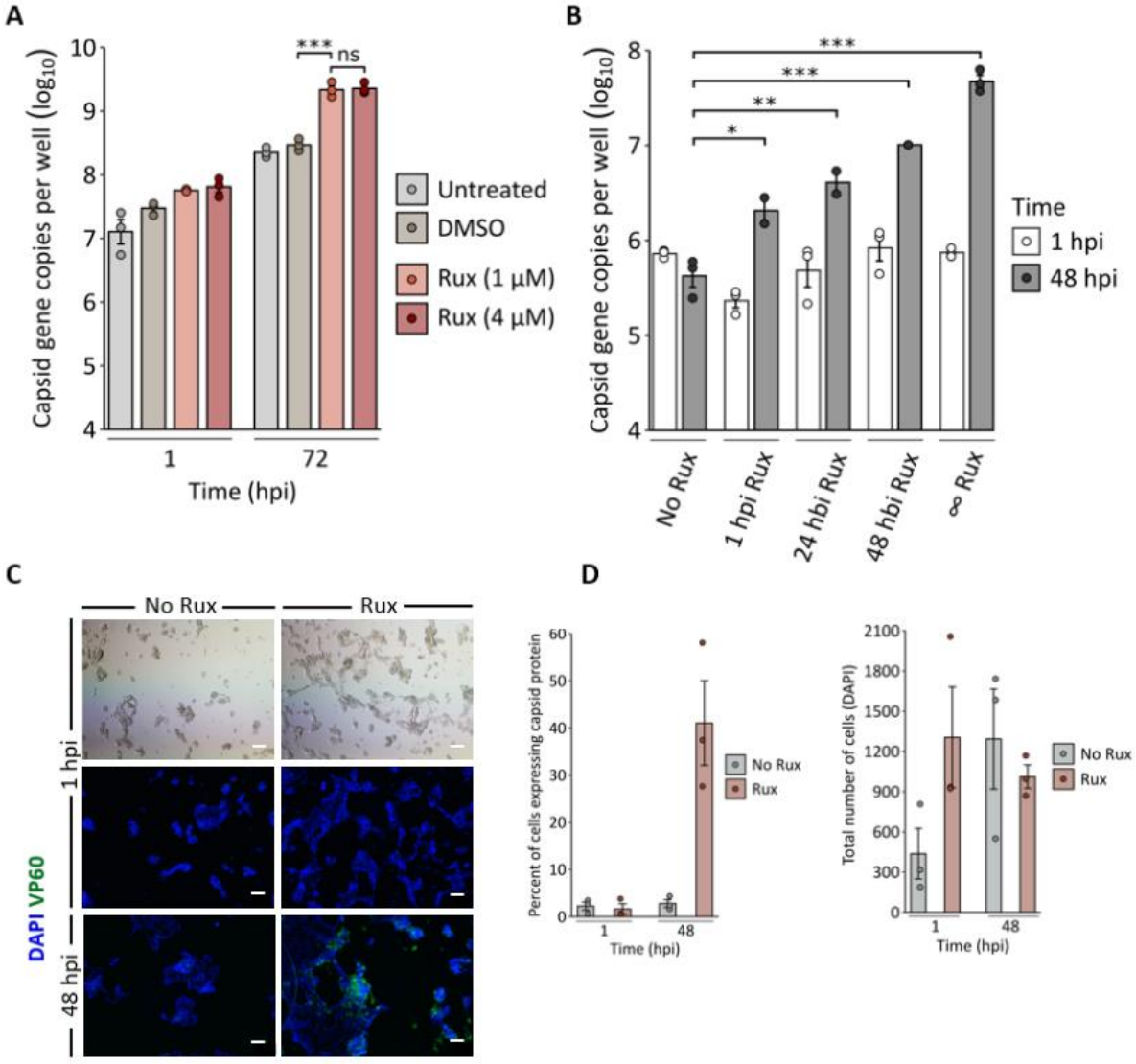
Figure 1.3: Passaging RHDV1 and RHDV2 in hepatobiliary organoid-derived monolayer cultures. Cells in the first passage were infected with semi-purified RHDV1 or RHDV2 (standard inoculum, ID_{50} titrated in rabbits Elizabeth McArthur Agricultural Institute) at a m.o.i. of 0.01. For passages 2 and 3, 100 µl of the supernatant from the previous passage was used as inoculum.



1.4.3 Interferon inhibitor enhances RHDV replication

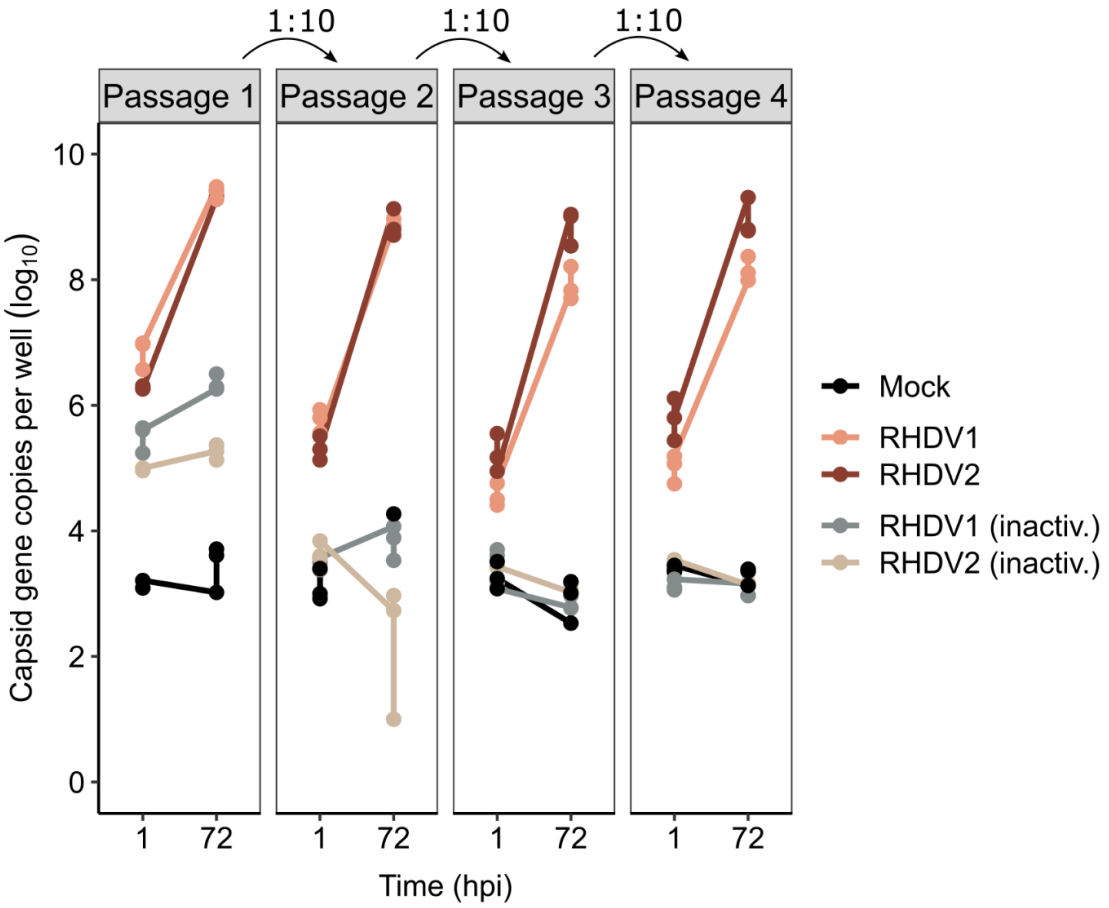
We investigated if virus passaging was hindered by the ability of infected cells to produce and release interferon, which would interfere with subsequent infections, especially after passaging. We found that the inhibition of interferon indeed resulted in a significant increase in RHDV2 viral titres in cells that were treated with the interferon inhibitor Ruxilitinib (Rux) at different concentrations prior to infection (Fig. 1.4).

Figure 1.4: Effect of Rux on RHDV2 replication in rabbit liver monolayers. (A and B) Rabbit liver monolayers supplemented with Rux 48 h prior to infection with 0.01 MOI RHDV2 at a MOI of 0.01. Virus replication was quantified using RT-qPCR at 1 and 72 hpi. Individual data points represent three biological replicates averaged from three technical qPCR replicates for each. Columns and error bars represent the mean values and the standard error of the mean, respectively. Significance is denoted by p-value calculated using ANOVA followed by Tukey's HSD test (*, p<0.001). (C and D) Immunofluorescence staining of infected rabbit liver monolayer cells in the presence or absence of Rux. Monolayer cells grown in chamber slides were infected with 0.01 MOI RHDV2 and immunostained with antibodies against the lagovirus capsid protein (VP60, green). The nuclei were counterstained with DAPI (blue). Scale bar is 100 µm.**



By further optimising protocols with the interferon inhibitor, we were able successfully passage RHDV1 and RHDV2 in organoid-derived monolayer cultures. This marks a significant advancement in organoid culture technology for studying these RHDVs in culture by effectively inhibiting the innate immune response in these cells. (Fig. 1.5). The results of this study have been submitted for publication to the *Journal of Virology* (Smertina et al., 2025). A draft of the manuscript is attached as a separate file.

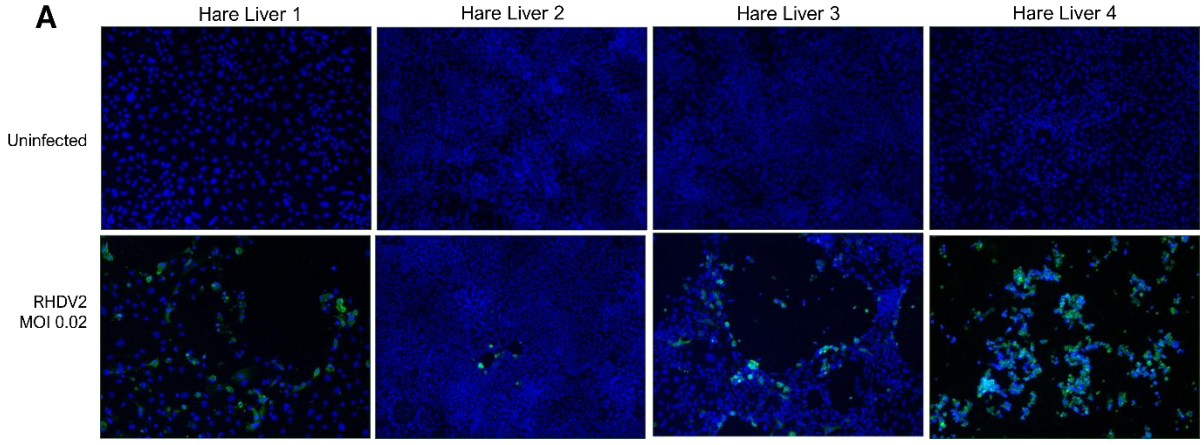
Figure 1.5: Passaging of RHDV in rabbit liver monolayers. The cells were infected with either RHDV1, RHDV2, heat-inactivated viruses (inactiv.), or mock-infected, and the capsid genome copies were quantified with RT-qPCR at 1 or 72 hours post infection (hpi). The supernatant aliquots from passage 1 were diluted 1:10 and used as inoculum for the next passage. Individual data points represent three biological replicates averaged from three technical replicates for each.

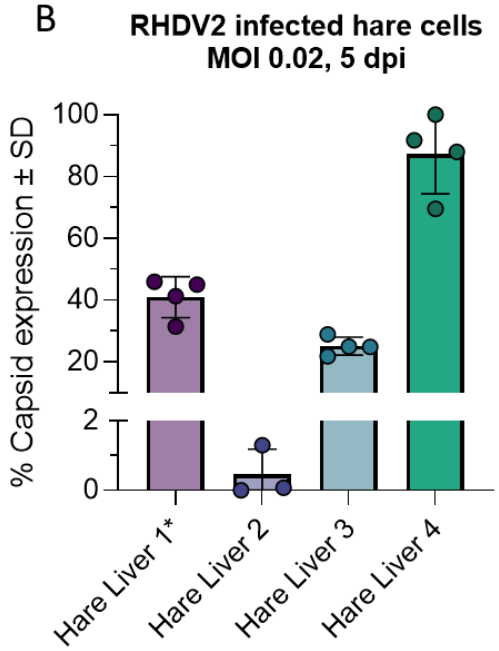


1.4.4 Hare liver organoid cultures with Ruxolitinib supports RHDV2 replication

Building on previous data with rabbit liver organoid cultures, four hare liver organoid cultures were successfully established. These hare liver organoid cultures were grown with Rux and infected with RHDV2 at an m.o.i. of 0.02, following the standardised batch-testing protocols. This approach was used to determine the most susceptible hare liver organoid culture that supported robust and productive RHDV2 infection and replication. All hare liver organoids displayed varying degrees in permitting virus infection and replication. We observed viral VP60 capsid protein expression (Fig. 1.6A) and genome replication in three of the four hare liver cells evaluated (Fig. 1.6). Hare liver 1, 3, and 4 organoid cultures supported RHDV2 infection while hare liver 2 organoid culture did not. Among these hare liver organoid cultures, hare liver 4 facilitated the most productive RHDV2 infection and replication with up to 87% of cells positive for RHDV2 VP60 capsid protein (Fig. 1.6B).

Figure 1.6: Expression of RHDV2 VP60 capsid protein in hare liver organoid cultures. Three independent hare liver organoid cultures were infected with RHDV2 at MOI 0.02 and at 5 days post infection (dpi), infected hare liver organoids cultures were fixed with 100% acetone. (A) Expression of RHDV2 VP60 capsid protein were visualised using indirect-immunofluorescence assay (IFA) with anti-VP60 primary antibodies. (B) Quantification of cells expressing RHDV2 VP60 capsid proteins in IFA. * denotes that the hare liver organoid culture was lost in the cryo-storage failure in late 2023.



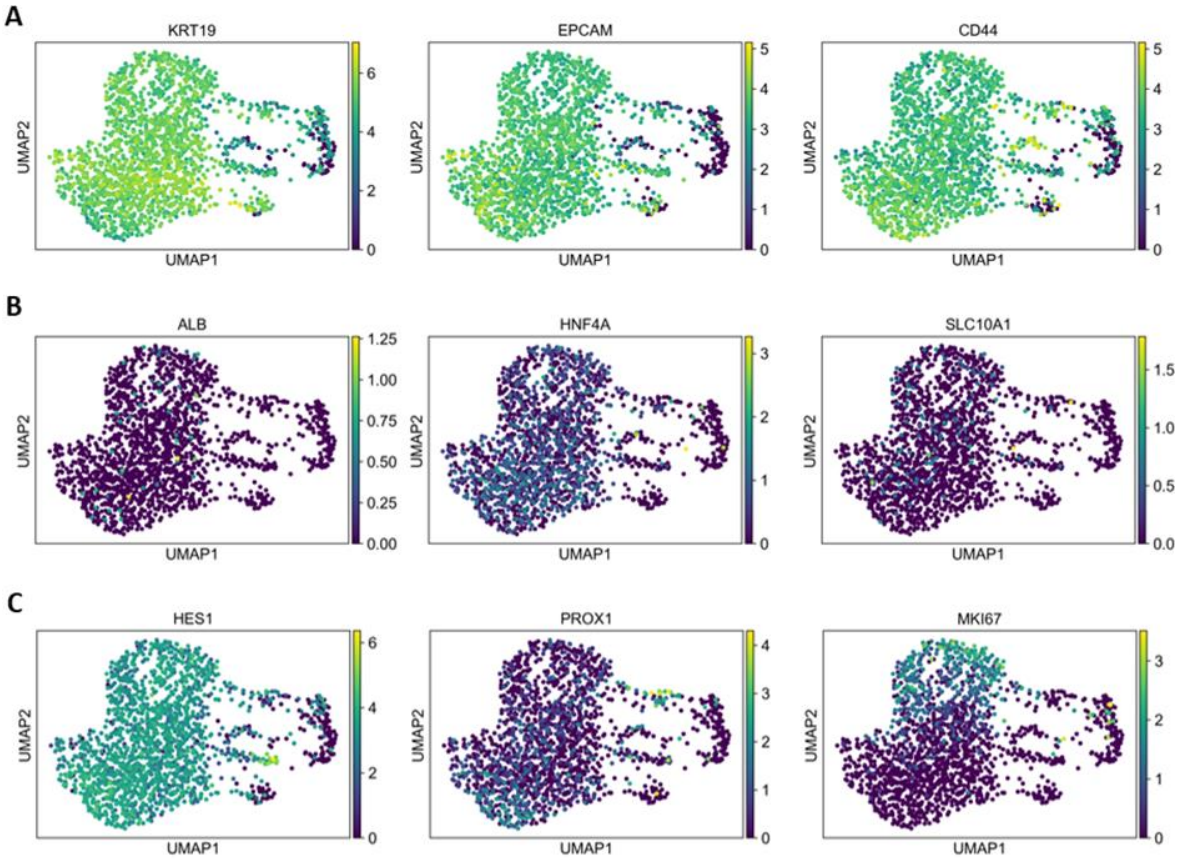


1.4.5 Single-cell sequencing to characterise rabbit liver organoids

We used single-cell RNA sequencing of selected liver organoid cultures to further characterise the cell composition using their gene expression profiles. Our results show increased levels of expression of cholangiocyte-specific genes compared to hepatocyte markers, which confirmed previous results (Kardia et al., 2023). EPCAM and KRT19 were highly expressed (Fig. 1.7A) and are a characteristic feature of cholangiocytes (Aizarani et al., 2019). High expression of epithelial progenitor cell marker CD44 was also observed, suggesting that the analysed cells are predominantly immature cholangiocytes. Mature hepatocyte markers (ALB, HNF4A, and SLC10A1) were expressed at low

levels (Fig. 1.7B), confirming that the monolayer cells are predominantly immature bile duct cells. The conclusion was further corroborated by the detection of high expression of the early development transcription factor HES1, which is essential for tubular bile duct formation (Kodama et al., 2004), and low expression levels of PROX1, responsible for hepatocyte proliferation and migration (Sosa-Pineda et al., 2000) (Fig. 1.7C). The observed clustering was attributed to the different stages of cell cycle rather than differential expression of cell type-specific marker genes. The presence of the proliferation marker (MKI67) in a small population of cells indicates at different cell cycle stage of that cluster (Fig. 1.7C).

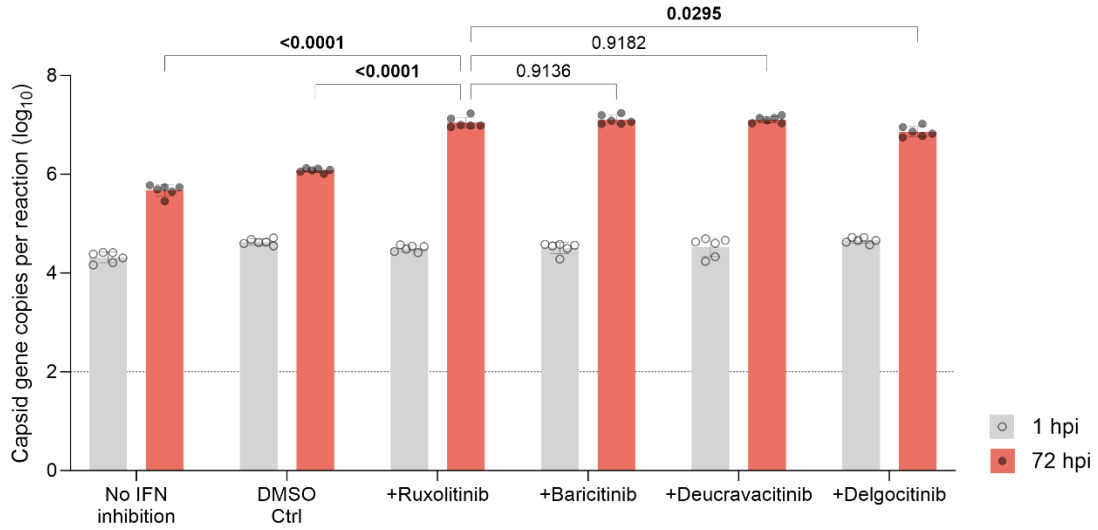
Figure 1.7: Uniform manifold approximation and projection (UMAP) plots of normalised marker gene expression in rabbit liver monolayers. Uniform manifold approximation and projection (UMAP) plots of normalised marker gene expression in rabbit liver monolayers. Each dot represents a cell, and the colour indicates the level of gene expression, with lighter colours indicating higher level of expression. (A) Expression of the cholangiocyte-specific markers cytokeratin-19 (KRT19), epithelial cell adhesion molecule (EPCAM), and epithelial cell marker cluster of differentiation 4 (CD44). (B) Expression of the hepatocyte-specific markers albumin (ALB), hepatocyte nuclear factor 4 alpha (HNF4A), and sodium-taurocholate co-transporting polypeptide (SLC10A1). (C) Expression of the early development transcription factor hairy and enhancer of split-1 (HES1), Prospero homeobox protein 1 (PROX1) and marker of proliferation Kiel 67 (MKI67).



1.4.6 Screening other Interferon Inhibitors

After successfully enabling RHDV1 replication and passaging of RHDV1 by adding Rux to the culture media, we explored alternative reagents with similar interferon inhibiting properties. Our goal was to identify compounds that could further enhance the replication capacity of our liver organoid platform. Three additional inhibitors were tested (Baricitinib, Deucravacitinib, and Delgocitinib). The results did not reveal any substantial improvements beyond what Rux offered (Fig. 1.8).

Figure 1.8: Replication quantification of RHDV2 in hare liver organoid cells in presence and absence of different interferon inhibitors. Capsid gene copies were quantified using qRT-PCR at two time-points. Infections conducted in presence of Rux showed significant increase in virus replication compared to control groups, however, none of the other three interferon inhibitors increased the level of replication. Statistical significance is denoted using p-values in bold following one-way ANOVA and Tukey's multiple comparisons.



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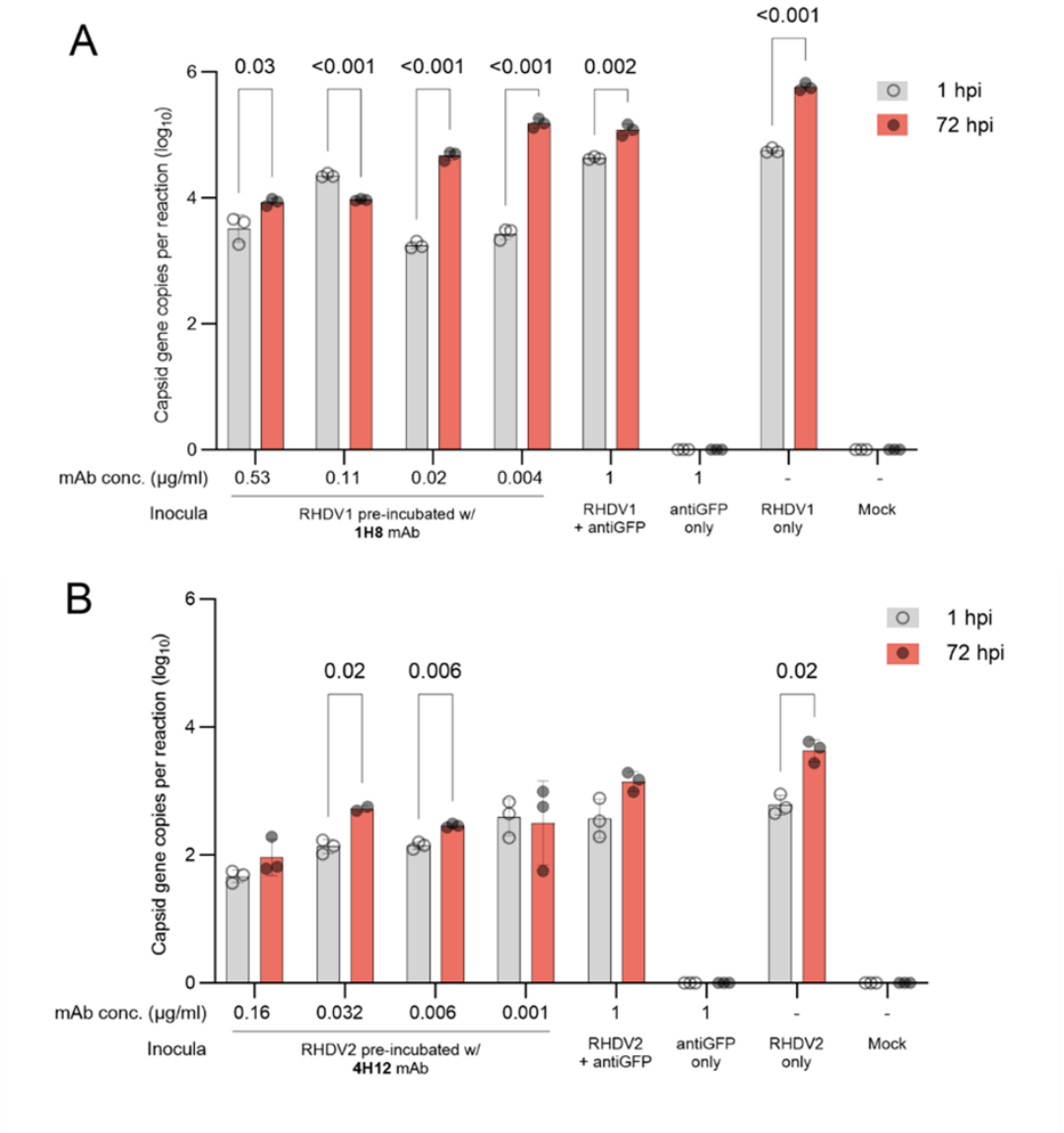
1.4.7 Finding the right antibody concentration for the selection experiments

Passaging of virus in the presence of monoclonal antibodies (mAb) and polyclonal antibodies (pAb) has commenced. In pilot experiments, mAb concentrations were titrated for RHDV1 and RHDV2, in preparation for future experiments aimed at serial passaging and the selection of escape mutants. (Fig. 1.9).

Figure 1.9: Initial passaging of RHDV1 and RHDV2 under monoclonal antibody selection pressure. This figure displays the outcomes of the first passage of RHDV1 (A) and RHDV2 (B) in rabbit liver organoid monolayer cultures, where the inocula for both viruses were pre-incubated with their respective monoclonal antibodies (mAbs) at various concentrations, as indicated. This preliminary experiment was conducted to determine suitable mAb concentrations for subsequent passages. Statistical significance is indicated by p-values, determined through one-way ANOVA and Tukey's multiple comparison tests.

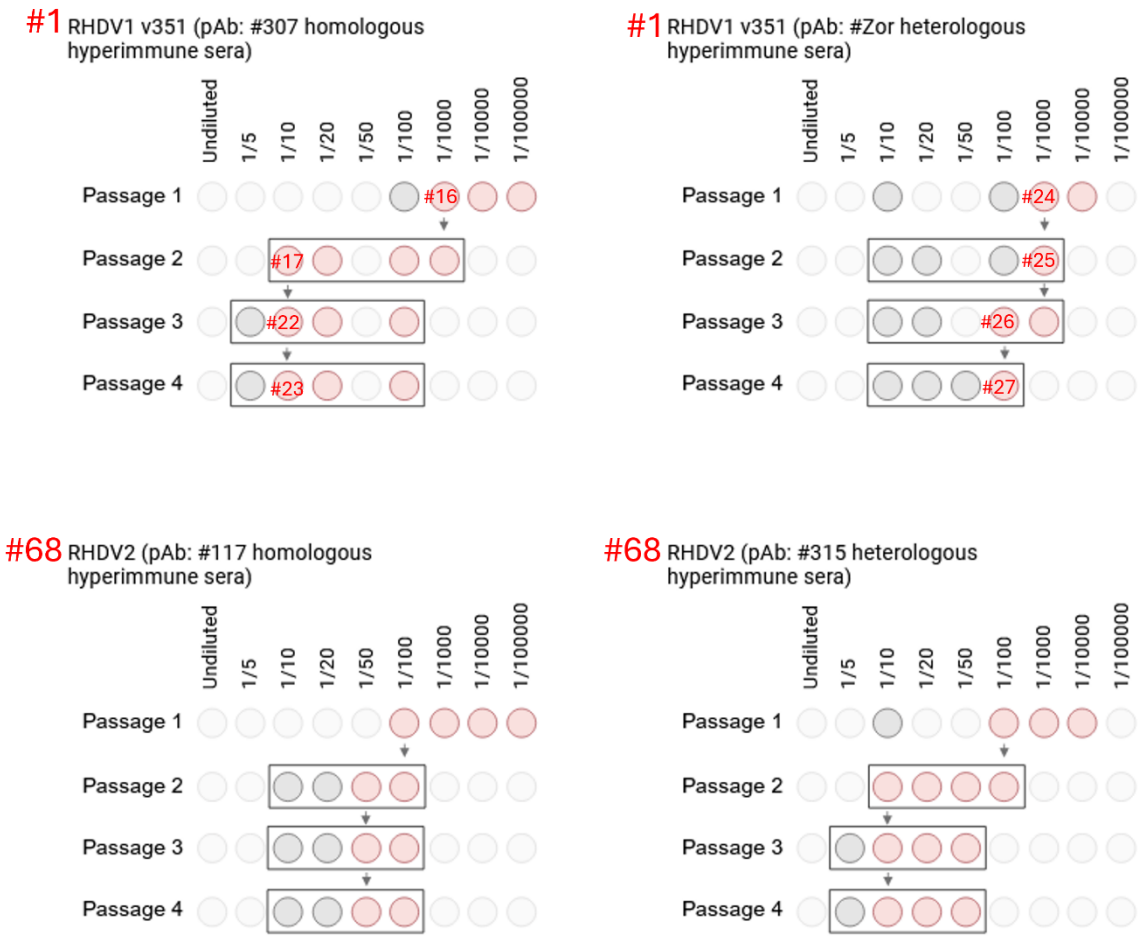


We also commenced preliminary investigations into passaging in the presence of polyclonal antibodies derived from the sera of reconvalescent rabbits that were infected with RHDV2. The virus was able to replicate at subsequently higher antibody concentrations (Fig. 1.10). While detectable virus replication was not observed above a 1:1000 serum dilution for RHDV1, concentrations of 1:10 dilutions were tolerated by the second passage for one polyclonal serum and at 1:100 by the third passage for a second polyclonal serum 1. Similar results were observed for RHDV2 (Fig. 1.10, lower panels). However, subsequent Sanger sequencing of the VP60 gene did not show any genomic

changes within the antigenic region. Further work is needed to determine if the virus was able to overcome higher concentrations simply by increasing the amount of virus transferred in later passages.

These preliminary experiments pave the way for future studies further optimising the selection process, including strategies to select with both monoclonal and polyclonal antibodies. Additional whole-genome virus sequencing will be required to analyse if any complementary mutations in other region might enable a higher tolerance to neutralising antibody concentrations.

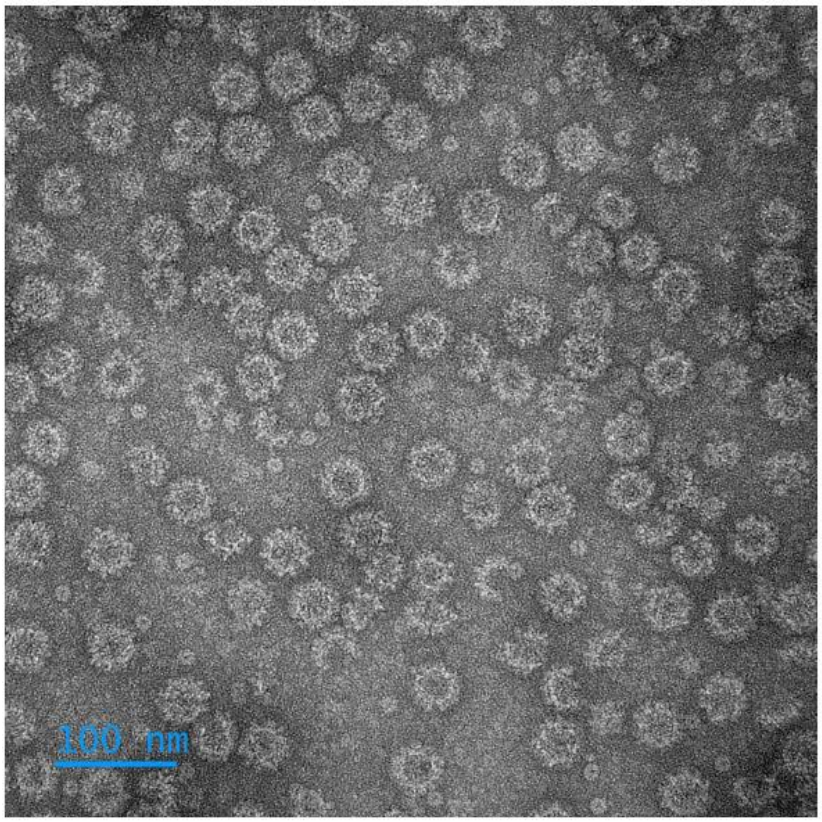
Figure 1.10: Passaging results for RHDV1 and RHDV2 under different antibody concentrations. Virus was passaged in the presence of serial dilutions of neutralising antibodies. For each passage, the virus was added to antibodies at four different dilutions. The arrows indicate the wells selected in the following passage. In the first two examples, the red numbers indicate virus samples from wells selected for further passaging. Selection was based on the ability of the virus to grow at the highest antibody concentration (i.e. the lowest dilution). For example, with RHDV1, sample #16 was selected after passage 1 to proceed to passage 2. Following passage 2, sample #17 was chosen, then sample #22 after passage 3, and finally, sample #23 was selected after passage 4 for the next round.



1.4.8 Generation of monoclonal antibodies (mAbs) against RHDV2

To strengthen our future work and to be less dependent on international suppliers, we generated our own set of RHDV2-specific mAbs for future research. For this process RHDV2 virus-like particles (VLPs) were produced using a commercially available Baculovirus expression system. The VLPs were purified by the CSIRO Manufacturing Business Unit and were used to generate a new panel of monoclonal antibodies at WEHI. VLPs are critical for this process, as they retain the three-dimensional structure of true RHDV2 particles (Fig. 1.11), while VLP preparations do not contain contaminations from an infected rabbit liver (Liu et al., 2012). In addition, commercial custom antibody facilities do not accept potentially infectious material as antigens for their antibody production services.

Figure 1.11: Transmission electron micrographs of purified RHDV2 virus-like particles (Photo: Joanne Lee, Australian National University Electron Microscopy Unit)



Two batches of mAbs were produced with a total of 23 mAbs produced. Our in-house ELISAs, immune fluorescence assays and Western blots were used to assess the ability to detect and distinguish between RHDV1 and RHDV2. A total of eight different mAbs with different reaction profiles were selected and are currently being produced and purified at the commercial antibody facility at WEHI.

1.5 Conclusion – Accelerator

After decades of research and failed attempts by research groups around the world, we were able to provide the first robust cell culture system for rabbit caliciviruses (RHDV1 and RHDV2). We were further able to optimise the organoid culture system by using an interferon inhibitor Rux, to enable

virus passaging over multiple passages. This is a breakthrough that will enable us and others to characteries aspects of the virus' life cycle that were previously unaccessible, without the use of animals. In addition, we demonstrate that organoid-derived cell culture systems can be used to analyse host-specificity, which will be invaluable to ascertain the safety of any putative future RHDV-based rabbit biocontrols. Furthermore, we commenced passaging experiments under selection with neutralising antibodies that can be built on in future studies.

Together with the production of a panel of monoclonal antibodies, this project component has successfully delivered a culture system, and the tools and protocols required towards an *in vitro* Accelerator system that can now be used to select for new and more potent RHDV variants to enhance the biocontrol of rabbits.

1.5.1 Key findings

- Rabbit liver organoid-derived cell culture system that supports RHDV1 and RHDV2 replication
- Hare liver organoid-derived cell culture system that supports RHDV2 replication
- Interferon inhibitor Rux enhances RHDV replication and enables passaging
- Liver-derived organoid culture systems can be used for host specificity assays
- Proof-of-concept for an *in vitro* accelerator platform
- Generated a panel of mAbs for future selection of variants by passaging under selection pressure.

1.5.2 Benefits to industry

The results of this project lay the foundation for an *in vitro* accelerator platform to future proof rabbit biocontrol. Our organoid-derived cell culture system is the first reported cell culture system to support RHDV replication and can be used for virus characterisation and evolution. With this system, we can provide a cost-effective platform to select for new virus variants that can enhance Australia's rabbit biocontrol and greatly reduce the use of laboratory animals in the process.

This new culture system will also enable functional studies aimed at understanding viral biology including factors determining host-specificity and virulence, as well as a range of other possible applications from vaccine production to studies on genetic resistance development against RHDVs in rabbits.

2. Receptor Studies

2.1 Background – Receptor Studies

Due to the initial challenges that we faced with the establishment of rabbit organoid systems and the passaging of viruses, we proposed a contingency plan aimed at the identification of the RHDV receptor. If the RHDV receptor for cell entry was identified, its gene can then be expressed in a standard cell line making it permissive for RHDV replication. A standard cell line is easier and cheaper to proliferate and maintain compared to the existing liver organoid system. This cell line would enable faster selection of immune-escape virus variants, vaccine production and improve molecular virology studies into RHDV pathogenicity mechanisms.

This sub-component of the project was included in 2022 as a means to drive forward the optimisation of the organoid system.

2.2 Objectives – Receptor Studies

- Explore different methodological avenues for receptor identification
- Determine ways to confirm any identified receptor candidates

All objectives of this project component have been met successfully.

2.3 Methodology – Receptor Studies

An experiment was conducted to quantify the number of organoid cells binding to virus like particles (VLPs) via the receptor). In this experiment, organoid cells were incubated with VLPs and unbound VLPs were washed off. Next, anti-VLP primary antibodies were applied, followed by fluorescently tagged secondary antibodies. Cells supporting binding were selected and quantified using a flow cytometer-activated cell sorter. Another approach undertaken was to quantify the permissible cell population. Organoids, infected with RHDV2 were subjected to fluorescence-activated cell sorting (FACS) to select for the cell proportion that is permissible for RHDV2.

To identify possible receptor candidates, a pull-down assay was performed with a trifunctional cross-linker (Sulfo-SBED). First, purified VLPs were covalently labelled with the cross-linker and incubated on the surface of the organoid-derived monolayers for virus-receptor interaction to occur. Next, UV light exposure was used to covalently link the receptor proteins to VLPs. The receptor-VLP complexes were then purified using a biotin group of the cross-linker, and the receptor candidates were identified using mass spectrometry. Candidate genes were cloned and transiently expressed in a non-susceptible cell line (rabbit kidney cells). These cells were then infected, and infection rates were measured using qPCR in a ‘gain of function’ assay where virus replication was expected if the expressed receptor enabled viral entry.

2.4 Results – Receptor Studies

Based on the results of the VLP binding experiment in the absence of Rux, only 5% of organoid cells supported virus binding. This observation was in line with the replication levels and

immunofluorescence staining studies previously that found only a small percentage of cultured cells to be infected (Kardia et al., 2023).

The cross-linking experiments and subsequent pulldown and mass spectrometry analyses identified a range of membrane proteins (Fig 2.1) that were further investigated as candidate receptors. Candidate proteins were membrane proteins involved in growth factor binding, cell adhesion, and membrane transport (Table 2.1).

Figure 2.1: Crosslinking pulldown assay results. Volcano plot visualising differentially expressed proteins, with the x-axis expressing the log2 fold-change and the y-axis depicting the statistical significance of the observed fold change. Highlighted in pink are the statistically significant outliers that were further investigated. This analysis also detects the viral capsid protein VP60 (the 'pull-down bait').

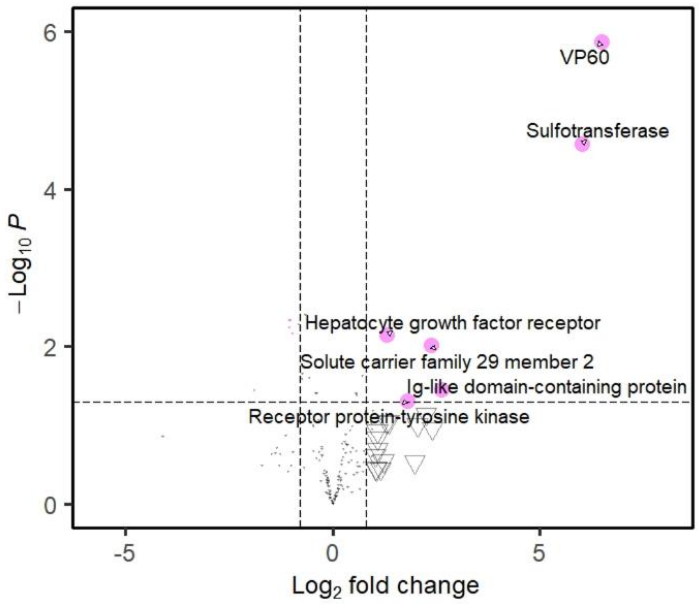


Table 2.1: Summary of identified potential receptor candidates.

Protein ID	Protein name	Protein function	Protein localisation	*p* value	Log2 fold change
Q09YN5	Hepatocyte growth factor receptor	Receptor tyrosine kinase	Plasma membrane	0.007	1.31
G1TJK6	Solute carrier family 29 member 2	Nucleoside transmembrane transport	Plasma membrane	0.010	2.37
G1SVN7	Ig-like domain-containing protein (CEACAM1)	Cell adhesion protein	Plasma membrane	0.035	2.62
G1THA5	Receptor protein-tyrosine kinase	Growth factor binding	Plasma membrane	0.049	1.80

Three receptor candidates, CEACAM1, tetraspanin and CD300lf were tested. To evaluate their potential role as RHDV receptors, the coding regions of these genes were cloned into an expression plasmid and transfected into the rabbit cell line RK13 that is non-permissive to RHDV infection. The transfected cells were infected with RHDV, and cells positive for the candidate receptor were tested for viral protein expression, which would indicate a successful infection. No RHDV protein expression or replication was detected in any of the transfected cells, concluding that these candidate proteins were not sufficient to act as RHDV receptors in these cells.

2.5 Conclusion – Receptor Studies

Although we identified potential receptor candidates using the methods in section 2.3, unfortunately expression in a continuous rabbit cell line did not result in RHDV replication. This suggests that either the identified candidates are not true RHDV receptors or that additional factors are necessary to facilitate RHDV cell entry and replication. Given these findings, we decided to intensify our efforts on enhancing RHDV replication and passaging in organoid-derived cell cultures, particularly after observing significant improvements with the addition of interferon inhibitor Rux as described above.

2.5.1 Key findings

- Susceptible cell population in organoids without the addition of interferon inhibitors was only 5%
- Several receptor candidates were identified using cross-link and pull-down assays
- Expression of several receptor candidates does not facilitate RHDV replication, indicating that these proteins are not sufficient to facilitate RHDV entry and replication

2.5.2 Benefits to industry

Although we did not manage to identify the receptor for RHDV or express it in a continuous cell line, we gained valuable insights into RHDV replication and its interactions with the cell surface. This knowledge will be useful in the future in characterising new virus variants selected through *in vitro* accelerated evolution. Additionally, the expertise developed through these methodologies provides a strong foundation for future research, strengthening the rabbit biocontrol pipeline.

3. Bioprospecting

3.1 Background – Bioprospecting

Bioprospecting in the context of biological control refers to the systematic and organised search for pathogens that could potentially be exploited for future rabbit biocontrol. Bioprospecting had been suggested previously as a strategy to supplement the rabbit biocontrol pipeline, using literature search approach to identify possible candidates (Henzell et al., 2008). This project chose an alternative approach which involved investigations of unexplained deaths or mass mortalities of rabbits and/or closely related species, both in Australia and overseas, with the aim to identify pathogens that could possibly be exploited for future biocontrol.

CSIRO's RHDV monitoring and surveillance program enabled us to test hundreds of samples of deceased rabbits annually to determine the cause of death and to study the epidemiology of different RHDVs in Australia. Since 2015, over 3500 samples have been analysed, revealing that approximately one third of samples are negative for RHDVs, leaving the cause of death unknown. It was anticipated that advanced sequencing technologies could help identify additional rabbit pathogens responsible for mortality, with the potential for use as biocontrol agents or biocides.

Other species closely related to rabbits within the order Lagomorpha present a possible reservoir for viruses that could be highly virulent in European rabbits. A textbook example for this is the *Myxoma virus*. First discovered in in *Sylvilagus* cottontail rabbits, myxoma virus is non-lethal in this natural host, but causes fatal infections in European rabbits, leading to its use as a biocontrol agent in Australia since the 1950s. Similarly, other viruses that naturally infect members of the order Lagomorpha without causing disease may exhibit increased virulence in European rabbits, presenting new opportunities for biocontrol.

Invasive European rabbits also pose significant economic challenges in South America, particularly in Chile. While *Myxoma virus,* the cause for Myxomatosis, is known to be circulating in those rabbit populations, little is known about the presence of lagoviruses. The detection and generation of genetic information of those viruses in Chile expands the knowledge of the genetic diversity in lagoviruses and can inform the development of new biocontrol agents based on these viruses.

3.2 Objectives – Bioprospecting

This project component aimed to investigate unexplained deaths in lagomorphs in Australia and overseas to identify pathogens that could be potential future biocontrol agents. This objective was successfully achieved through collaborations with scientists in Chile and the United States of America. While we identified pathogens not known to be present in Australia and discovered new viruses and virus variants overseas, none were deemed to be suitable candidates for future biocontrol. Results of this project component were published/submitted in various papers (Jenckel, Hall, et al., 2021; Jenckel, Smith, et al., 2021; Jenckel et al., 2022; Smertina et al., 2024; Jenckel M. et al., 2025).

The aims of the bioprospecting were to:

- Investigate unexplained rabbit deaths in Australia from tissue samples collected through a 10-year long RHDV monitoring program
- Establish international collaborations to source lagomorph tissue samples for the identification of new caliciviruses or other rabbit pathogens

- Dissemination of results through publications in scientific journals

All objectives of this project component have been met successfully.

3.3 Methodology – Bioprospecting

With the advent of next generation sequencing technologies, sequencing has become significantly more affordable, enabling applications that were previously unfeasible. Metatranscriptomics is the unbiased total RNA sequencing of a sample that can be used to determine the composition of microorganisms including viruses. This technique allows for the detection of potential pathogens and establishes microbial baselines in healthy individuals. We employed this technology to identify new biocontrol agents in Australia and elsewhere.

CSIRO maintains an extensive rabbit tissue database with more than 3500 samples from CSIRO's monitoring program and routine testing of deceased rabbits for RHDV collected over >10 years. Approximately one third of these samples are negative for RHDV. From this sample bank, we selected 48 samples, focusing on clusters of unexplained rabbit deaths and applied metatranscriptomics to identify potential causes. Additionally, the host RNA sequencing data that is inevitably generated, provided insights into the pathways associated with immune response, further supporting our findings by studying host responses to active infections present at time of death.

This method was also applied to samples acquired through collaborations with the Texas Tech University (Lubbock, Texas) and the US Geological Survey's National Wildlife Health Center that were either from apparently healthy individuals or deceased lagomorphs other than European rabbits (*lepus* and *sylvilagus* species) with unknown causes of death.

In collaboration with researchers in Chile, we screened rabbit samples for the presence of rabbit calicivirus variants with a broadly reactive Lagovirus-PCR followed by a metatranscriptomic approach for positive samples.

RT- and RT-qPCRs were utilised to screen additional samples for the presence of selected pathogens or to validate sequencing results.

Additionally, a serological Luminex assay was developed to detect Hepatitis E virus antibodies, allowing for the assessment of prior infection status in tested samples.

3.4 Results – Bioprospecting

3.4.1 Onshore Bioprospecting – Pathogen profiling in Australian rabbits

In the onshore bioprospecting, we were able to identify a range of known rabbit pathogens that can contribute to the death of a rabbit. As the presence of a pathogen does not necessarily mean that it was the cause of death, an extended analysis of the RNA host data was used to determine if immune and pathogen response pathways were upregulated to further support the role of the infection in the animal's death.

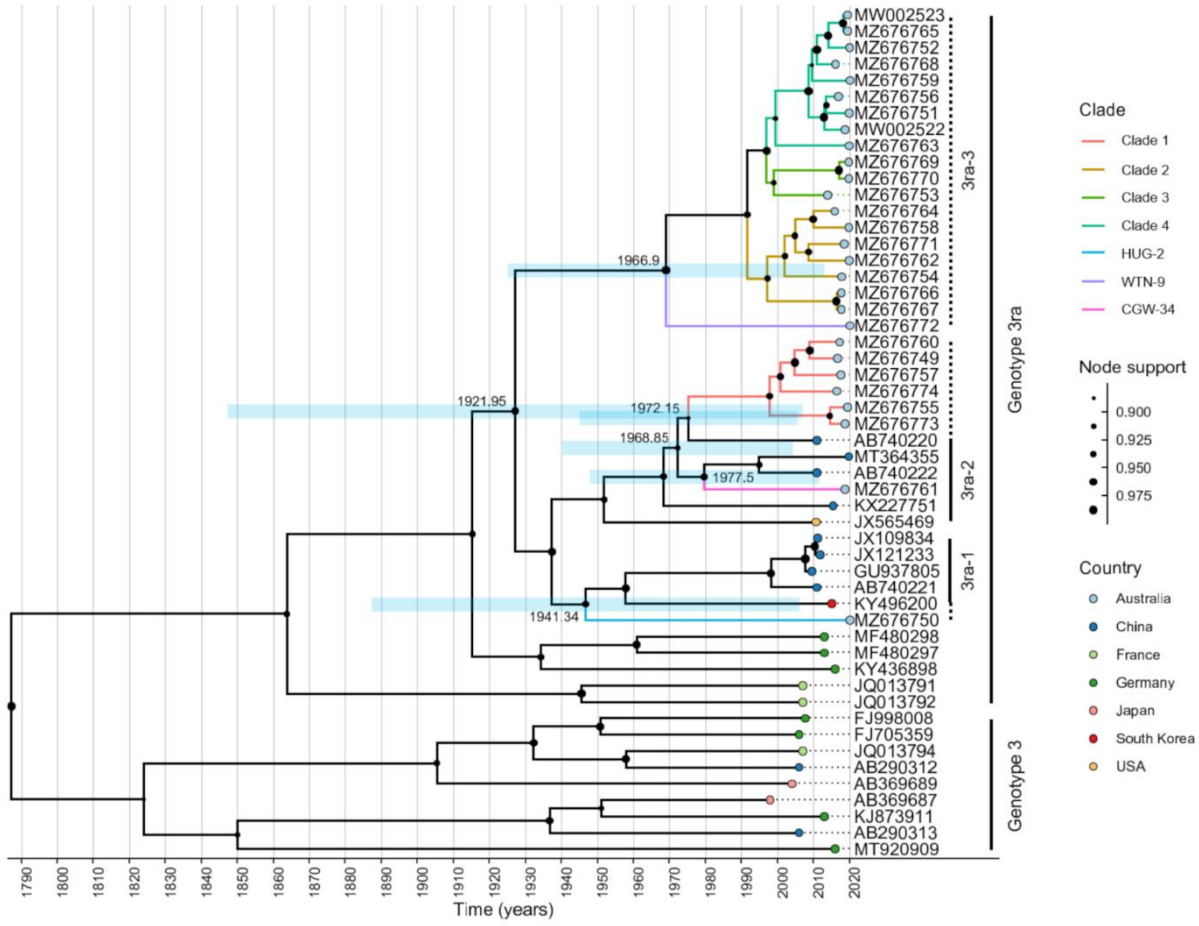
We identified transcripts from several *Clostridia* species, *Pasteurella multocida, Pseudomonas sp.*, and *Eimeria stiedae* in liver samples of several rabbits that had died suddenly. All of these bacterial pathogens are known to infect rabbits and are capable of causing fulminant disease (Harcourt-Brown et al., 2020). In addition, we identified *Hepatitis E virus* (HEV) and the yeast *Cyniclomyces* in some samples, both of which are not usually associated with severe disease. In one third of the

sequenced liver samples, no infectious agent could be identified. Additional host transcriptome analysis provided further insights to distinguish between pathogenic microbes and commensals or environmental contaminants, by assessing if the rabbit's immune genes were upregulated at time of death. Interestingly, three samples in which no pathogen could be identified showed evidence of upregulated host immune responses, while immune response pathways were not upregulated when *E. stiedae, Pseudomonas*, or yeast were detected. In summary, although no new rabbit pathogens were identified, this study established a protocol for future investigations into rabbit mortality events. The findings were published in the journal *Transboundary and Emerging Diseases* (Jenckel et al., 2022).

The major finding within this study was the first detection of Hepatitis E virus (HEV) in Australian rabbits. Hepatitis E virus is a potential zoonotic pathogen that was not known to be present in Australian rabbits. Genetic analysis showed that the identified viruses were closely related to other known lagomorph HEV strains with a 96% sequence identity to HEV strains in Europe. These findings were published separately in the *Australian Veterinary Journal* (Jenckel, Hall, et al., 2021).

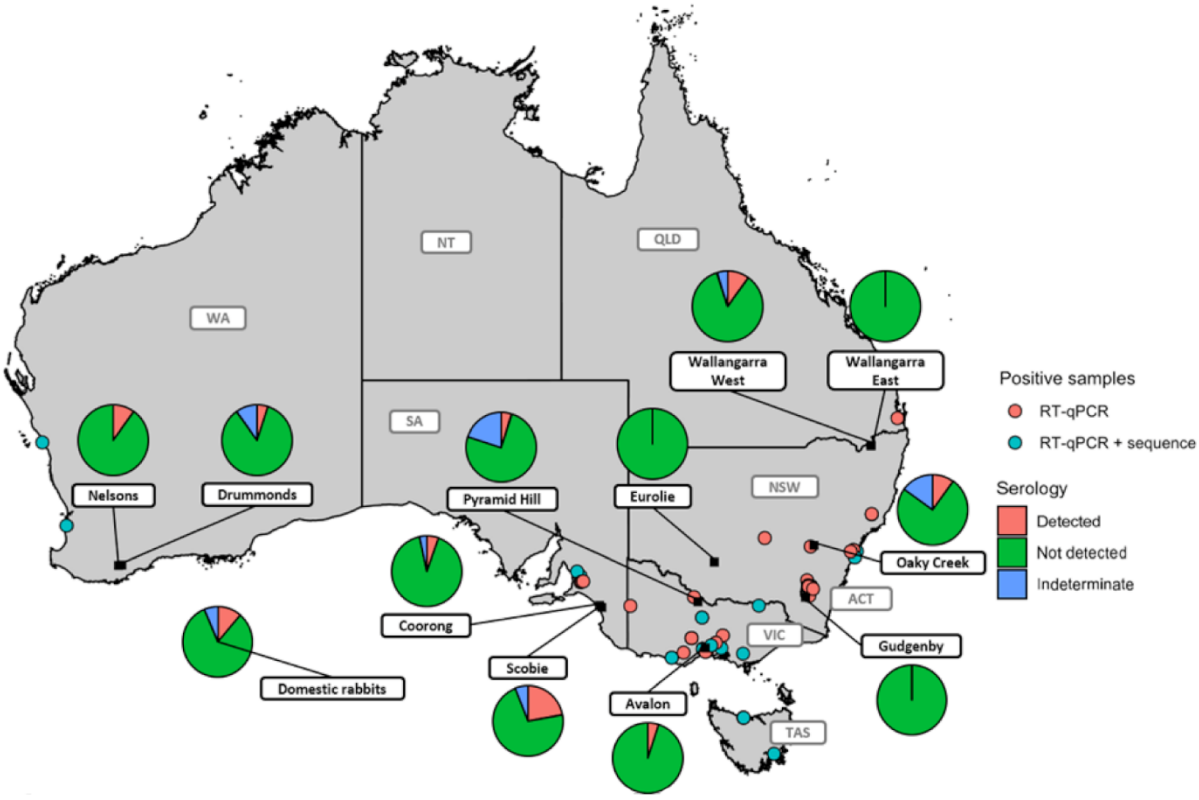
Considering the high abundance of wild rabbits in Australia, the potential zoonotic risk and overlapping tissue tropism with RHDV (both target the liver), a subsequent study was undertaken to evaluate the prevalence of HEV in Australian rabbits. A total of 1635 liver samples from CSIRO's rabbit sample collection were screened using an established RT-qPCR assay, resulting in the detection of 58 positive samples with the subsequent acquisition of 28 HEV genome sequences. The time resolved phylogenetic analysis suggested multiple introductions of HEV into Australia, ranging from the 1940s to the 1970s (Fig. 3.1). However, the overall lack of genomic information for HEV in publicly available databases hindered the calculation of more precise time frames.

Figure 3.1: Time resolved phylogenetic analysis of Australian and international HEV sequencing of Genotype 3 and 3ra. Time-resolved phylogenetic analysis of Australian HEV-3ra, global HEV-3ra and representative global whole-genome sequences of genotype 3 HEV. Genotype 3 reference sequences were based on those used by Smith et al. (Smith et al., 2013). Tips are coloured according to the country of origin and node support is indicated by the size of the filled circles at internal nodes. Branches that lead to Australian sequences are coloured based on the PrimalScheme assay used for amplification. Blue horizontal bars correspond to the 95% highest posterior density (HPD), with the median indicated at the relevant node. The x-axis is given in years. Clade labels indicate genotype 3 non-rabbit HEV sequences and the rabbit-specific HEV-3ra sequences, including the previously defined subgenotypes 1 and 2. Dotted line clade labels show the extension of existing subgenotypes and newly defined one.



The seroprevalence for HEV was tested at 11 sites across Australia and 80 serum samples from domestic rabbits collected between 2012 and 2020. The results ranged from 0–22%, depending on the location. Samples from domestic rabbits showed a 12% prevalence. The sample locations and serological results are shown in Fig. 3.2; the figure shows the origins of RT-qPCR positive samples and where a genome sequence was generated.

Figure 3.2: HEV distribution in Australia-positive tissue samples based on the RT-qPCR assay and sampling sites for HEV serology. Dots refer to samples from dead rabbits that tested positive in the RT-qPCR assay, colours indicate whether a near-complete genome sequence was obtained. Black squares depict the sampling sites for serological analysis, and pie charts display the number of detected, not detected and indeterminate samples. WA—Western Australia, NT—Northern Territory, SA—South Australia, QLD—Queensland, NSW—New South Wales, VIC—Victoria, TAS—Tasmania, ACT—Australian Capital Territory.



Overall, results show that HEV is widespread in Australian rabbits. However, due to a similar tissue tropism for HEV and RHDV, it remains unknown whether coinfections of HEV and RHDV would have a synergistic or antagonistic effect on the virulence of the RHDV infection and whether HEV activity could generate windows of opportunity or rabbit biocontrol with RHDV. The results were published as a feature paper in the special issue "Hepatitis E and the One-Health Aspect: A Threat to Mankind and Animality?" of the journal Pathogens (Jenckel, Smith, et al., 2021). All objectives of this project component have been completed.

3.4.2 Offshore Bioprospecting

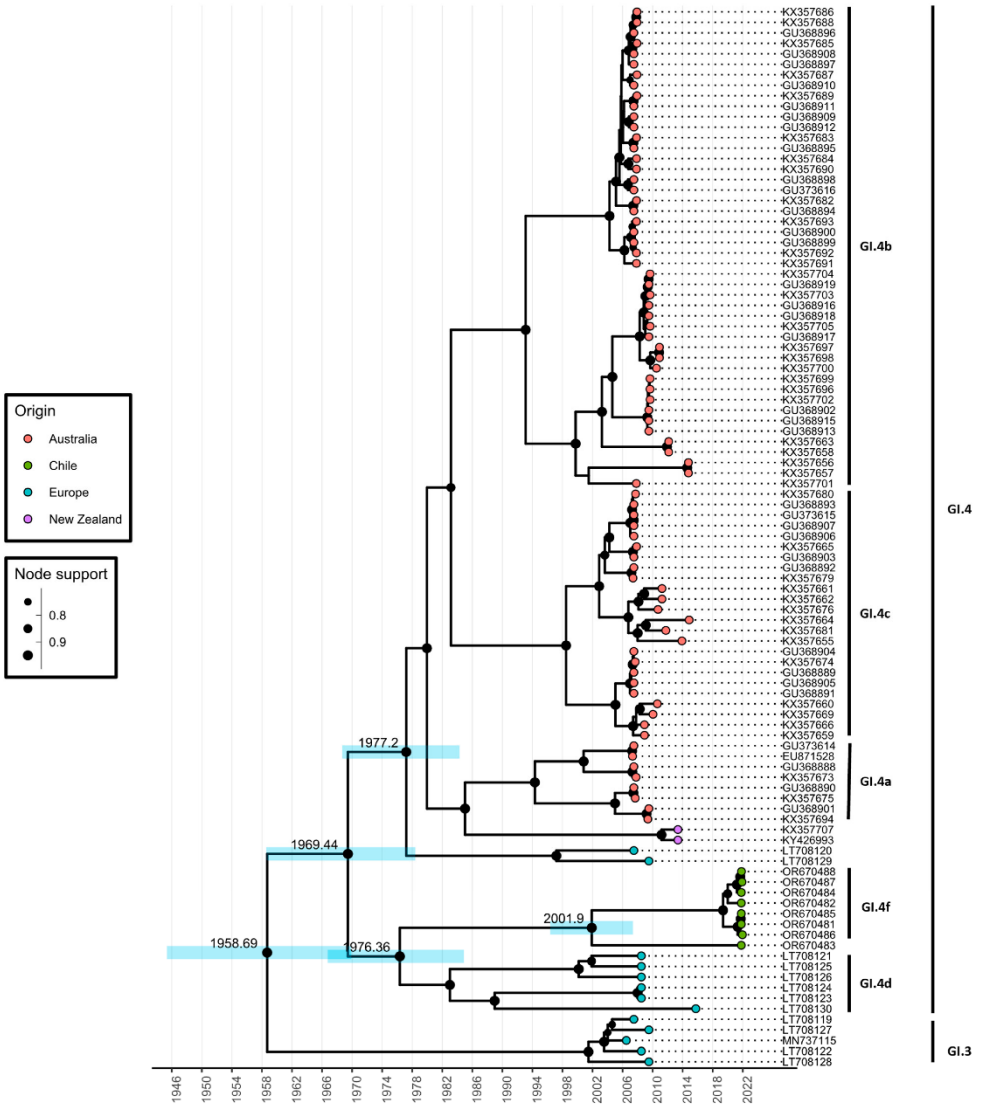
To investigate potential rabbit pathogens overseas we established collaborations with the USGS National Wildlife Health Center (USA), the Texas Tech University (USA) and the Pontificia Universidad Catolica de Chile (Chile).

Like in Australia, European rabbits are an introduced species in Chile, where they cause ecological and economical damage. In the 1950s, *Myxoma virus* was released to control the rabbits in Chile, and the virus is still circulating across Chile. Although the presence of *Myxoma virus* in Chile is

known, there had been no information on the presence of rabbit caliciviruses in Chile. A total of 113 paired liver and duodenum samples that were collected between October 2021 and June 2022 in three different locations in Central Chile were tested for the presence of caliciviruses. All liver samples tested negative while eight duodenum samples tested positive. These were subsequently used for a total RNA sequencing approach as described above. For all eight samples, a complete or near complete genome sequence could be generated. Phylogenetic analysis revealed the presence of two distinct RCV strains. Analysis of the non-structural genes showed the closest relationship to Australian RCV variants, while RCV variants originating from Europe were found to be the closest relative for the structural genes.

Temporal analysis (Fig. 3.3) showed that the Chilean RCV diverged in the 1970s from European strains. An introduction into Chile can be assumed around the same time or later. Interestingly, this timeframe overlaps with the estimated introduction of RCV into Australia (Mahar et al., 2016).

Figure 3.3. Time-resolved phylogenetic analysis of the RCV capsid coding sequence (green dots). The *x* axis represents a time scale in years. Median Time to Most Recent Common Ancestor (TMRC) values for GI.4f are shown at the nodes and blue node bars demonstrate 95% highest posterior density (HPD) intervals. Node support is indicated by the node size. Only node support values above 0.70 were considered as well supported and are displayed. The GenBank accession numbers are used as sequence names. Vertical lines on the right side indicate RCV variants and genotypes.



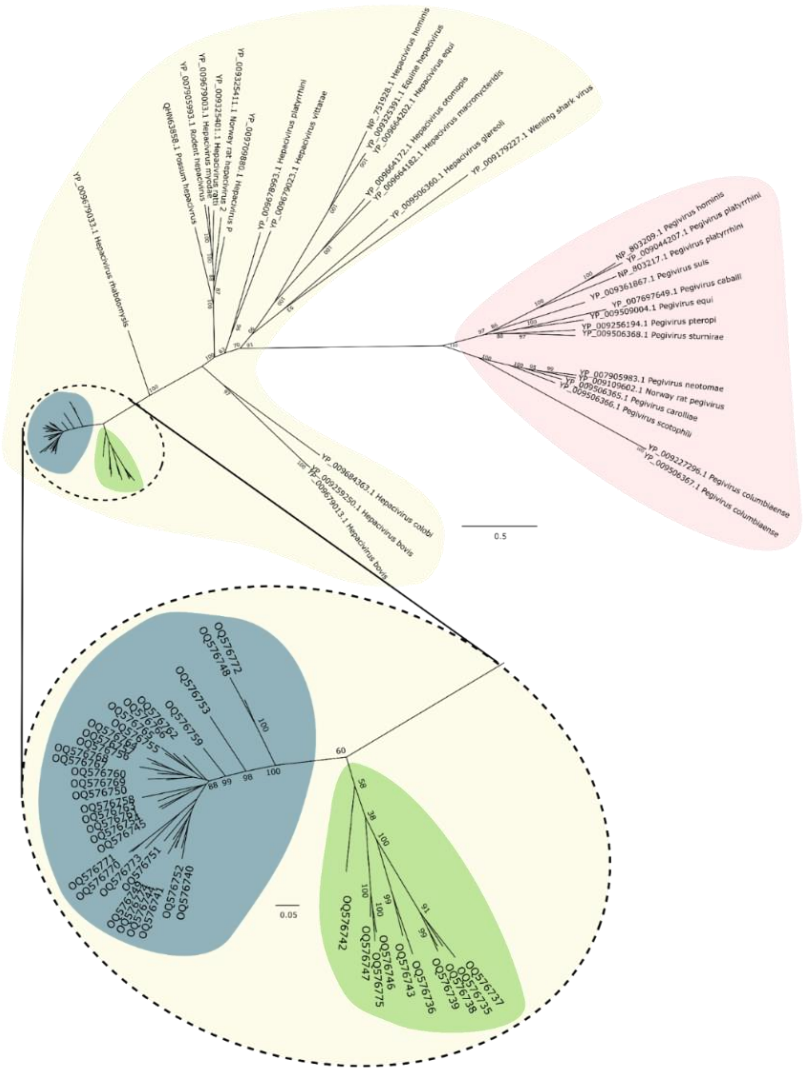
A coincidental finding during this analysis was the detection of a rabbit astrovirus. Rabbit astrovirus can cause gastroenteritis; however, it is also found in healthy animals. In this case, it was also not thought to have caused disease as the animal from which this sample was collected did not show any signs of disease. Phylogenetic analysis showed that the virus sequence clustered with other known rabbit and marmot astroviruses showed a 91.6% nucleotide identity to the rabbit astrovirus reference strain (NC_025346). The results of this study have been published in the special issue "Rabbit Viral Diseases" of the journal *Viruses* (Smertina et al., 2024).

North America is home of several native lagomorph species that are related to the European rabbit. This is of particular interest, because the first rabbit virus used for biocontrol in Australia, *Myxoma virus*, originated in *Sylvilagus* (cottontail) species, native to the Americas, and does not cause disease in its native host. We therefore investigated the microbiome/virome of native North American lagomorphs to search for new biocontrol agents. For this purpose, we used two different sample sets. The museum of the Texas Tech University harbours an extensive tissue collection. Based on the available lagomorph samples, we created 48 paired liver and lung sample pools, covering a total of 150 individuals, to investigate the virome and potential pathogens in native North American lagomorphs. The second type of sample were liver samples from deceased animals sent to the USGS

National Wildlife Health Center for RHDV2 testing. The samples all tested negative for RHDV2 and therefore presented a good opportunity to explore if other organisms and viruses potentially cause disease and death in North American lagomorphs. Species sampled included eastern cottontails (*Sylvilagus floridanus*), desert cottontails (*Sylvilagus audubonii*), mountain cottontails (*Sylvilagus nuttallii*), brush rabbits (*Sylvilagus bachmani*) and black-tailed jackrabbits (*Lepus californicus*).

The majority of the 20 genera of pathogens identified in this study were either known rabbit pathogens (e.g., *Pasteurella*), commensal or opportunistic bacteria (e.g., *Enterobacter*, *Corynebacterium, Escherichia*) or likely environmental contaminations (e.g. *Culex, Aspergillus, Phoma*). Of note was the detection a hepacivirus. Hepaciviruses are single-stranded RNA viruses that can infect a wide range of animals. The most prominent member of the genus is the Hepatitis C virus that can cause acute and chronic hepatitis in humans with initially no or mild symptoms. So far, hepaciviruses have not been known to infect lagomorphs. Like RHDV and HEV it infects the liver. In our study, we identified 67 samples that were positive for the newly discovered hepacivirus, including those from 48 eastern cottontails, 18 desert cottontails and one mountain cottontail. Out of those positive samples, we were able to assemble 41 complete or near-complete genome sequences. Comparison to known hepaciviruses showed that the newly identified virus has only approximately 50% sequence identity on nucleotide and protein level. The closest known relative is the *Hepacivirus I*, isolated from an African grass mouse. Further analysis of conserved regions showed that the lagomorph hepacivirus constitutes a new hepacivirus species. Phylogenetic analysis of the RdRp protein sequence of the newly generated hepacivirus and references from related hepaci- and pegiviruses shows not only that the lagomorph hepacivirus is only distantly related, but that they also form two distinct clusters based on the host it was isolated from (Fig. 3.4).

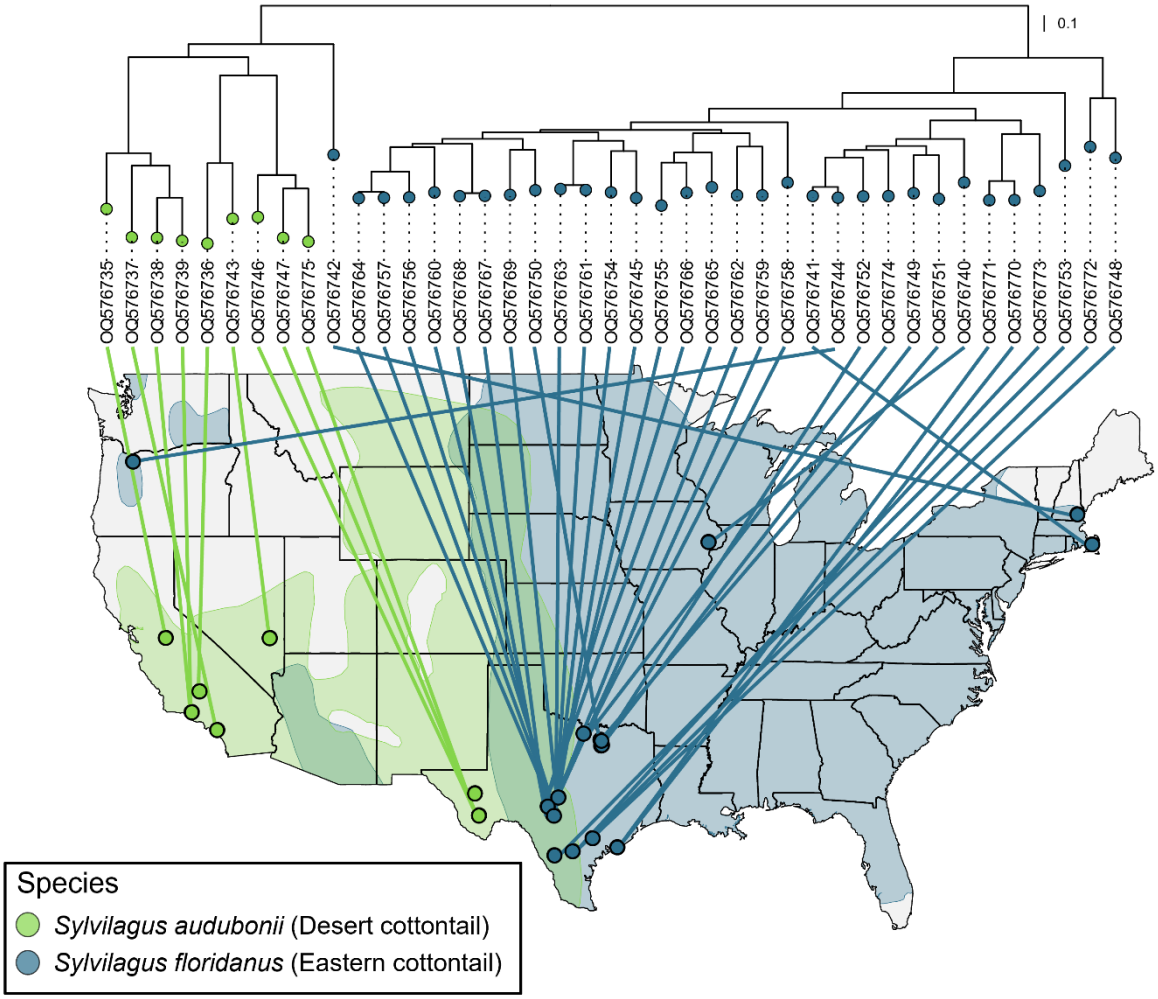
Figure 3.4: Unrooted phylogenetic tree of the RdRp protein sequence of hepaci- and pegiviruses. Phylogenetic analysis of the RdRp protein of the newly identified lagomorph hepacivirus (n = 41) and reference sequences from the genus *Hepacivirus* (n =19) (yellow) and Pegivirus (n = 14) (pink). The maximum likelihood tree was calculated using IQ-Tree (v 2.2.0.5) with the best-fitted model (LG+I+G4) and 1000 ultra-fast bootstraps. Scale bar represents substitutions per site. The lagomorph hepacivirus clades are coloured by dominant host species they were isolated from. Blue: Eastern cottontail (*Sylvilagus floridanus*), Green: Desert cottontail (*Sylvilagus audubonii*).



Further phylogeographic analysis of the lagomorph hepacivirus showed that the clustering of viruses correlates with the habitat and distribution of the respective host (Fig. 3.5).

Figure 3.5: Phylogeographic analysis of North American lagomorph Hepacivirus ORF1 sequences originating from eastern (*S. floridanus*) and desert cottontails (*S. audubonii*). A maximum likelihood phylogenetic tree was estimated using the best-fitted model (GTR+F+I+G4) in IQ-TREE (v 2.2.0.5) with 1000 ultra-fast bootstrap replicates. The tree was rooted at the branch leading to Hepacivirus I (NC_038428). Only the subtree containing the lagomorph hepacivirus is shown. Colours refer to the different cottontail species the virus was extracted from. Lines connect the position in the tree and the corresponding geographic location in the US. The natural habitat of

both cottontail species according to the IUCN red list is displayed as coloured areas. *S. floridanus* – Eastern cottontail; *S. audubonii* – desert cottontail.



In addition to a new hepacivirus, we identified a picorna-like virus in one sample and a tetnovirus in two samples. Based on available data, it is most likely that these are both environmental contaminations and do not infect the lagomorphs. The results of this study have been submitted for publication to the journal *Virus Evolution*. The draft of the manuscript is attached as a separate file. All objectives of this project component have been completed.

3.5 Conclusion – Bioprospecting

The detection of rabbit HEV and the identification of a new lagomorph hepacivirus demonstrated that an unbiased metatransriptomics approach is the tool of choice to investigate the pathogen landscape of rabbit populations in Australia and overseas. In addition to standard metagenomics analysis, we also show that the RNA host data can be utilised to provide evidence that identified pathogens contribute to disease or death of the respective animal. Although none of the viruses discovered in this project were deemed suitable for rabbit biocontrol, their presence in lagomorph populations raises several questions that might affect future biocontrol decisions. While hepatitis E virus and the newly discovered lagomorph hepacivirus do not seem to cause severe disease in rabbits, they have the same tissue tropism as RHDV and could therefore cause synergistic or antagonistic effects with RHDV infections, for example though elevated innate immune responses. This is of special interest in the context of Hepatitis E virus, because our study shows a wide

distribution of the virus in the Australian rabbit population. Further studies could investigate how prior or concurrent Hepatitis E virus infections influence natural RHDV infection and biocontrol outcomes.

As for the other virus discoveries, the potential of lagomorph hepacivirus to cause virulent diseases in European rabbits is unknown and would require exploratory studies. While the RCV variant detected in Chilean rabbits is benign, the genome sequence still offers valuable information that can be utilised in the RHD-Accelerator project component to better understand and optimise new virus variants. Virulent RHDV is also believed to have evolved from benign RCV-like ancestors, and therefore, any region with endemic RCV warrants close observation for the potential emergence of another virulent rabbit calicivirus. The project also strengthened our international collaboration in the Americas, which will be highly valuable in the event of any newly emerging lagomorph viruses.

3.5.1 Key findings

- First detection of Hepatitis E virus in rabbits in Australia
- HEV is widely distributed in wild and pet rabbits in Australia
- Wild rabbits in Australia are affected by a range of different microorganisms, many of them known rabbit pathogens
- The analysis of host mRNA can provide evidence that an identified organism contributed to disease or death of an individual
- Chile is home to at least two different strains of no-pathogenic rabbit calicivirus
- We identified a new hepacivirus species in North American lagomorphs with unknown virulence potential in European rabbits

3.5.2 Benefits to industry

In this part of the project, we established international collaborations in North and South America. These contacts are of importance for future work, because they will enable us to get access to samples in case of mass mortality events in either native North American lagomorphs or introduced European rabbits in South American. This will benefit future bioprospection approaches of novel biocontrol agents.

The outputs from this project component demonstrate that metagenomics approach is highly successful in pathogen identification. The method can be easily adapted to other investigations where the causative agent of death is suspected to be a pathogen, but routine diagnostics fail. Furthermore, this method can be used to identify pathogens in other pest species that could be potentially used for vertebrate pest management.

4. Long term biocontrol pipeline

4.1 Background – Long term biocontrol pipeline

Genetic biocontrol technologies (including but not limited to so called “CRISPR-Cas gene drives”) are currently being discussed worldwide as a potentially more humane approach to managing invasive species. These technologies could, for example, skew the sex ratio toward male offspring, ultimately leading to population decline (Esvelt et al., 2014). Proof-of-concept of this technology has been demonstrated in mice as a vertebrate model system (Gierus et al., 2022).

Beyond technical feasibility and challenges such as regulatory approval and social acceptance, the success of these approaches depends on the ability of novel genetic traits to penetrate a population. This process is influenced by the species biology, population structure, generation time, and migration patterns of any given species in any given environment. In addition, for the precision gene editing in genetic biocontrol, a complete and accurate information on the target sequence is essential. While some studies have examined the genetic structure of Australian wild rabbit populations, comprehensive data on their genomic diversity remains limited. Therefore, there was a clear need to combine population genomics and genetic modelling as a first enabling step to inform the development of Australian-specific genetic biocontrol and predict their potential safety and efficacy.

The findings of this project component will guide further research into candidate genes suitable for gene drive applications while also providing preliminary data for regulatory bodies to evaluate the efficacy and risks associated with targeted genetic interventions.

4.2 Objectives – Long term biocontrol pipeline

The objective of this project component was to sequence a wide range of Australian wild rabbits across different time periods and geographic locations. By comparing this sequence data with existing European rabbit sequences, the goal was to identify locally fixed alleles that could serve as potential targets for gene drives. A locally fixed allele is genetic target sequence that is present in 100% of the target population and enables the spread of the gene drive but occurs at a lesser frequency in non-target populations, resulting in gene drive failure in non-target populations. These locally fixed alleles, which represent an important genetic safety mechanism for gene drives, would then be used in modelling to assess their suitability for facilitating population collapse.

- Whole genome sequencing of diverse (time and geographic location) rabbit samples
- Identification of locally fixed alleles as target genes for population control
- Modelling of gene drives and suitability for genetic biocontrol
- Publication of results

All objectives of this project component have been met successfully, with the exception of the publication of results. A draft manuscript is close to submission and attached as a separate file.

4.3 Methodology – Long term biocontrol pipeline

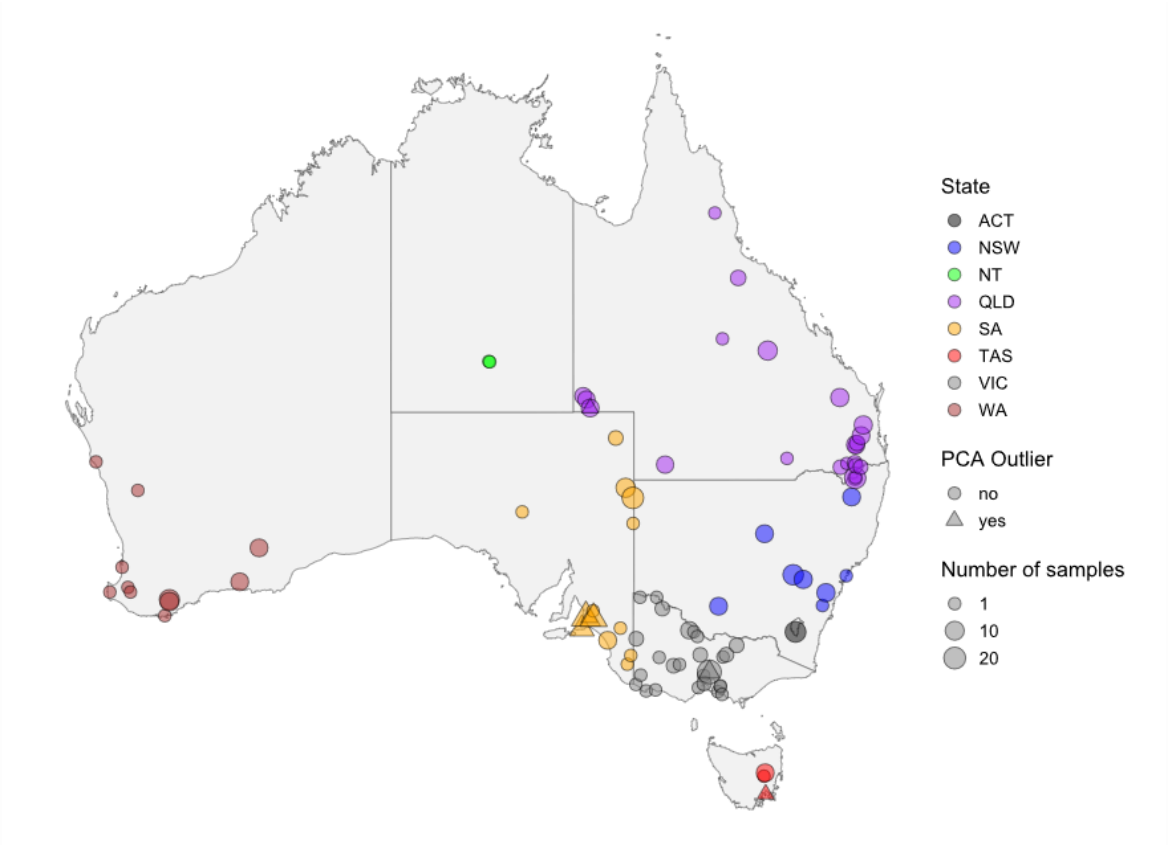
Our objective was to identify potential guide RNAs (gRNA) within gene regions and assess variations across the targeted Australian rabbit population. Guide RNAs are a crucial component of the CRISPR-Cas9 system that act as a molecular guide directing the Cas9 protein to a specific DNA sequence for editing. Variations within specific parts of the gRNA sequence, particularly the protospacer adjacent

motif (PAM) and within 4 to 7 bp upstream of PAM can severely impact the binding of CRISPR-Cas and its effectiveness in gene drives. A population genomic survey was needed to narrow down candidate gRNAs that could effectively target the Australian rabbit population while minimising the risk to non-target populations.

To achieve this, we sequenced whole genomes of over 360 individual wild rabbits from 114 locations across Australia (Fig. 4.1), aiming for 5 to 8 times sequencing coverage to identify individual genotypes and characterise the genetic variability and population structure of wild rabbit populations in Australia. Additionally, we incorporated data from 180 rabbit genomes provided by European collaborators from the UK and Portugal, as well as six domestic rabbit sequences provided by our collaborators in the UK.

Eighty-nine Australian low coverage samples were re-sequenced to boost sequencing depth to an average of 5 times.

Figure 4.1 Sampling sites for Australian States and Territories. Dots indicate the locations where rabbits were sampled for inclusion into the analysis. Triangles indicate outlier samples as identified by PCA, see Fig. 4.2.



We utilised the pipelines from the 'Gene Drive Utility and Risk Determination' (GUARD) platform developed in-house at CSIRO for pest insect populations. This bioinformatics platform enabled the identification of unique genetic target sites for potential genetic biocontrol tools. These target sites were selected based on their presence in all individuals within the target population (e.g., Australian wild rabbits) while being absent or found at low frequencies in non-target populations (e.g., wild rabbits in Europe). Our study specifically focused on genes related to reproduction. Subsequently, the theoretical spread of any such tool could be modelled based on the genetic variability and

population structure also obtained by genomics sequencing. The GUARD platform encompasses the following steps: (1) Generating whole genome variant for every sampled individual, (2) identify all possible gRNA across the whole genome for every individual, (3) narrowing down gRNAs that have shared variation in key positions of the gRNA across all samples from the targeted population, (4) further narrowing down to gRNA within exons or 5' untranslated regions in genes, (5) population dynamic models based on the allele frequencies of the sampled populations to enable evaluation of efficacy in target population and risk of impact to non-target populations under different sets of conditions.

We focussed on genes related to reproduction, including ovarian follicle development, spermatogenesis, oocyte maturation, fertilization, and sperm-egg recognition. Additional genes were included based on previous studies relevant to reproduction or gene drive strategies for population control (Carneiro et al., 2015; Champer et al., 2018; Gardela et al., 202; Clark et al., 20240).

4.4 Results – Long term biocontrol pipeline

4.4.1 Population genetic analysis

Low-quality samples (<0.5x coverage) for Population Genetic Analysis were excluded, leaving 402 samples (283 from CSIRO, 113 from Europe and 6 domestic rabbits). As described in previous reports and studies (Alves et al., 2022), when compared to European rabbits, most Australian rabbits appear quite genetically homogeneous. However, there were some outliers, mostly from South Australia. These outliers may have domestic origins, as suggested by their proximity on the PCA (Fig. 4.2), shared ancestral states observed in admixture plots (Fig. 4.3).

Figure 4.2: PCA analysis of whole genome data from Australian and European rabbits. Outlier Australian sample locations are named on the plot (below PC2 < -0.05). Colours represent the possible different ancestral genetic clusters (9 clusters identified, labelled as clusters 1-9). On the left are Europe, followed by South Australia outlier (boxed), domestic (boxed) and all other Australian populations.

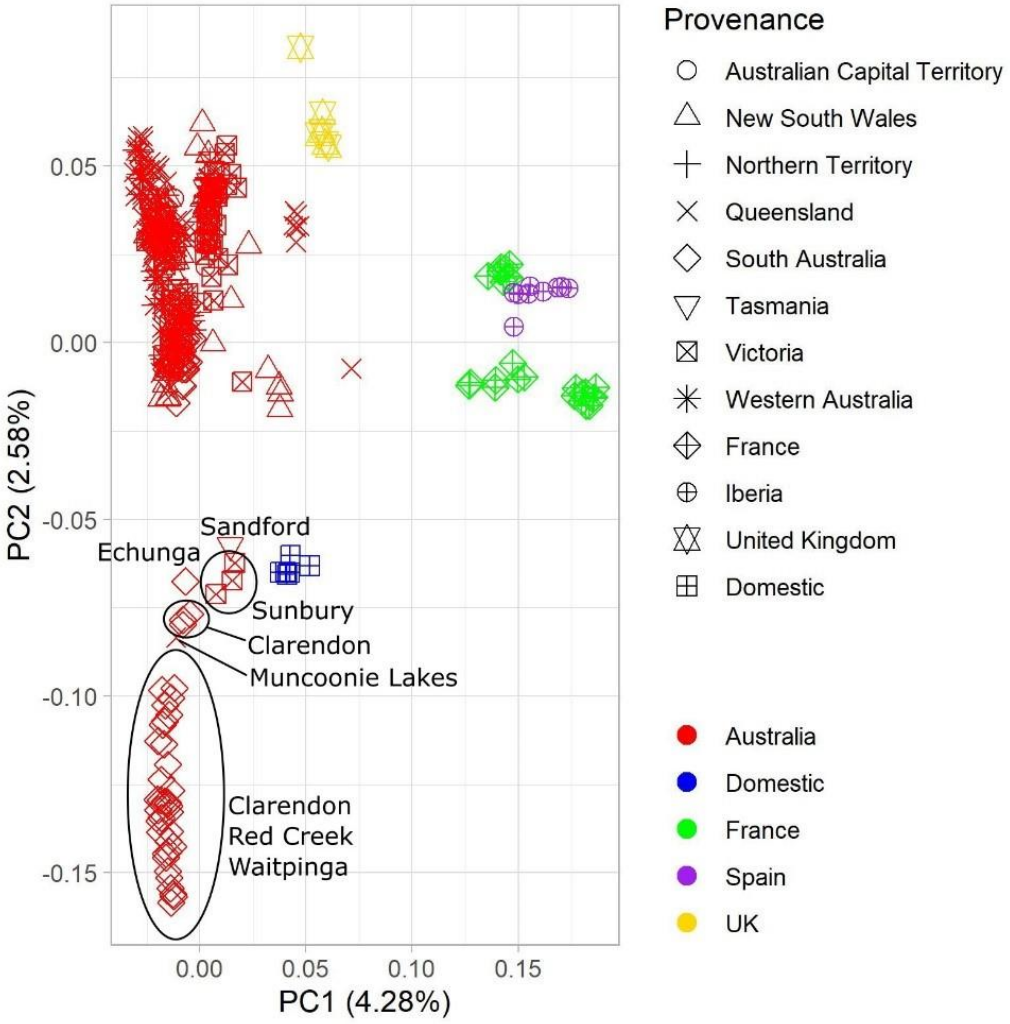
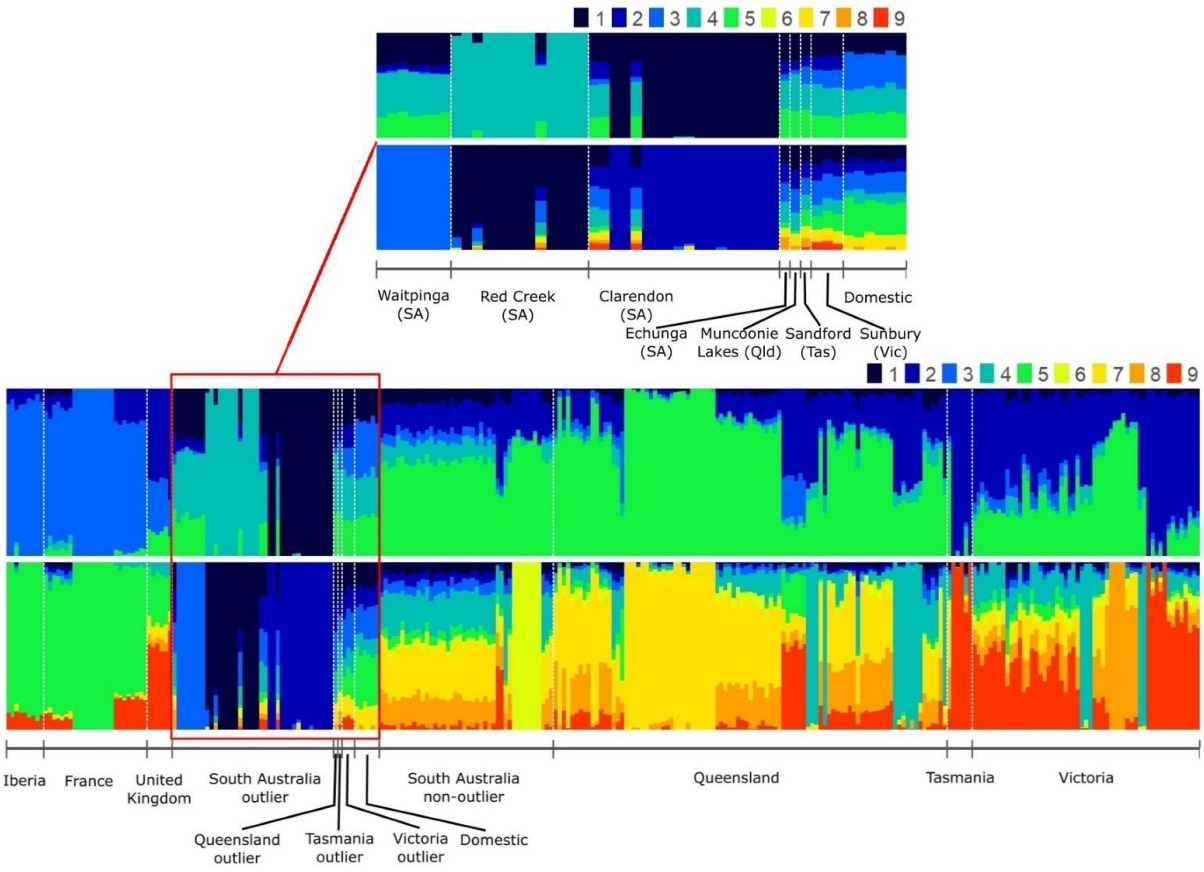


Figure 4.3: Admixture analysis of rabbit samples. Colours represent the possible different ancestral genetic clusters (9 clusters identified, labelled as clusters 1-9). The first three on the left are Europe, followed by South Australia outlier (boxed), domestic (boxed) and all other Australian populations. We found the optimal number of clusters were K = 5 and 9 (top and bottom figures). South Australian outliers clustered separately from the rest of Australia. Domestic rabbit contained some shared clusters (shared colours) with South Australian outliers, however, for any given South Australian outlier rabbit vs domestic rabbit, the proportion shared cluster was not particularly high.



4.4.2 Identification of gRNAs

Previous analyses, using an older reference sequence (OryCun2.0), identified 51,366,115 single nucleotide polymorphisms (SNPs) and 11,733,589 insertions or deletions (INDELs). However, due to low coverage in a significant portion of data, the variant filtering led to the removal of >70% of identified variants. Applying the locally fixed allele approach (Sudweeks et al., 2019; Oh et al., 2021) to identify CRISPR-Cas9 targets, we found over 69,000 gRNAs that were fixed across all of Australia but not fixed in Europe (with a frequency of <95%). Among these, 1,218 gRNAs were predicted to affect gene expression in at least 800 genes (out of 20,000 protein coding genes) including 60 gRNAs targeting 38 reproductive genes from our gene list. Of the 60 gRNAs, only 33 gRNAs had at least one other gRNA within 500-bp vicinity. Additional sequencing of low coverage samples plus the availability of a new reference sequence (mOcyCun1.1) increased the total number of gRNAs but reduced the number of potential reproductive genes, most likely due to improved annotation (Table 4.1).

Table 4.1 – Number of gRNAs identified as possible CRISPR-Cas9 target within the Australian population. The candidates were chosen on the basis that in European populations the gRNA haplotype exist at a frequency <95%. Numbers in brackets refer to number of genes targeted by gRNAs

Rabbit genome reference	Total	Within Genes	Possible Reproductive Genes	Multiplex within Genes	Multiplex within possible reproductive genes
OryCun2.0	69,960	1,218 (800)	60 (38)	648 (272)	33 (14)
mOryCun1.1+89 samples	106,177	2,158 (1302)	58 (38)	1,199 (478)	26 (11)

All bases in the PAM site and +4 to +7 upstream of the PAM in identified gRNA must have a minimum sequencing depth of 2x in at least 50% of sampled target populations.

We identified the Dynein Axonemal Heavy chain 17, (DNAH17) and Nanos C2HC-type zinc finger 2 (NANOS2), based on their roles in reproduction, as promising targets for gene drive. DNAH17 had been implicated in spermatogenesis in mice, human and rat (Chen et al., 2021; Zhang et al., 2019) with single evidence of fertile female homozygous for DNAH17 mutation. Mutations in NANOS2 have been implicated in male sterility in mice, boar, goat and cow, with evidence of fertile females in mice (Ciccarelli et al., 2020). One gRNA for each gene (Table 4.2) was used for modelling.

Table 4.2 – Candidate gRNA selected for theoretical gene drive spread modelling based on the mOryCun1.1 reference genome

Scaffold	Start	End	Phase	Sequence*	Gene
NC_091448.1	3,067,724	3,067,745	+	AACCT[G]AACAAC**CTGA**CGT**C[G]G**	DNAH17
NC_091449.1	11,136,815	11,136,836	+	GGCTGGTGGGGC**[T]GGG**GCC**CGG**	NANOS2

*In red is the PAM site, in bold black is the +4-+7 upstream of PAM and in square brackets are mutations found in the dataset

The results of this component are currently prepared for publication, a copy of the draft manuscript is attached as a separate file (Yeap et al., 2025, manuscript in preparation).

4.4.3 Population dynamic modelling

For the population dynamic modelling, we accounted for a potential risk of migration (i.e. wild rabbits getting into Australia), though in reality, Australia's strict biosecurity laws would keep this risk close to zero. At zero migration, the gene drive would consistently suppress the target population without posing a risk to non-target populations.

To model gene drive spread in Australian and European rabbit populations, we assumed a constant migration rate and applied the gene drive models (SIEGE), developed at the University of Melbourne (Camm & Fournier-Level, 2025). The models integrated sequence information and population genetic parameters from our study. SIEGE was chosen for its ability to incorporate gRNA sequence variation and model its impact on CRISPR gRNA gene drive conversion efficiency. This type of model incorporates stochasticity, key variables in CRISPR-based gene drive such as fitness cost, selective pressures and gene drive dominance. It also accounted for population variables such as inbreeding coefficients, migration and population size. Conversion efficiency rates (probability of conversion to

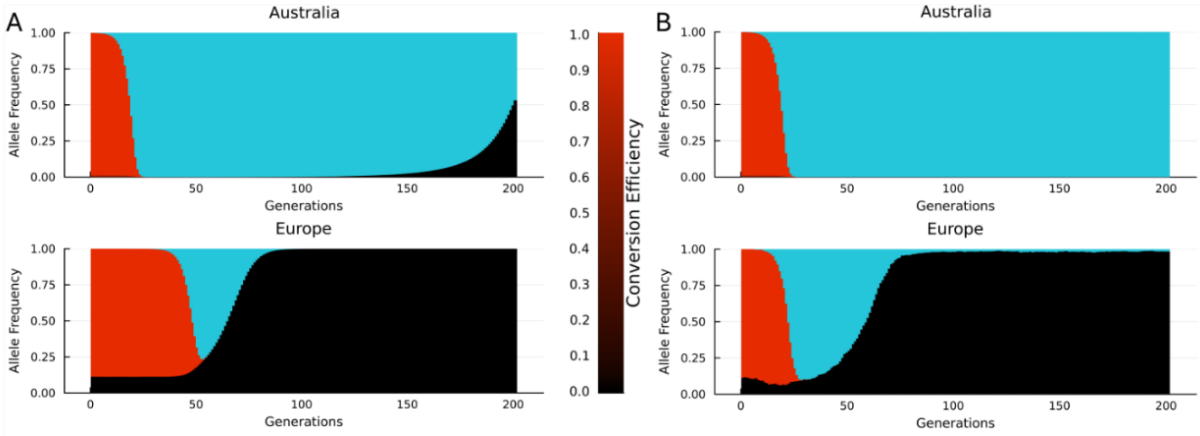
gene drive haplotype) were determined based on reported single-base conversion effects (Hsu et al., 2013).

We tested scenarios where the effect of the gene drive was optimal: (1) sex bias effect was perfect and impaired one sex completely, (2) the sex bias effect was dominant to wild type, (3) the fitness of the impaired sex was zero, (4) low fitness cost to the unaffected sex. Migration rates were set to approximately 5 x 10^{-6} (5 individuals for every 1 million per generation) and were estimated based on pairwise fixation index (F_{st}) between Australia and Europe. We also assumed that the gene drive induces a deleterious sex bias effect, such that only one sex will remain if all individuals harbour the gene drive haplotype thus causing a population crash. We set a constant large effective population size for Australia (target population) at approximately 1 million and varied the population size of the non-target European rabbits, one at the same population size as Australia and another at a significantly reduced number, 1000 to reflect an endangered status. For simplicity reasons we did not allow spontaneous mutations to occur in the current models. We simulated an observation period of 200 generations.

We identified three haplotypes for the DNAH17 gRNA (mOryCun1.1 scaffold NC_091448.1 at positions 2,824,978-2,824,999), the most common was found in both Australia and Europe at >98% and 88.8% frequency, respectively and the candidate gRNA was designed based on this. The second haplotype in Australia was rare and was mostly susceptible to conversion (>60% chance), while the last one found in Europe was fully resistant because of a single mutation on the second base of the PAM site (Table 4.2).

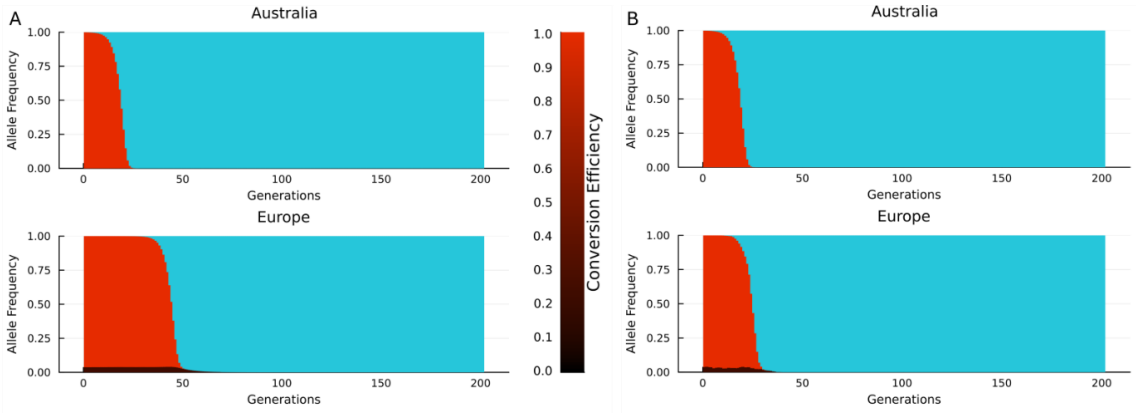
Based on the assumptions above, the DNAH17 gene drive will be effective in Australia, with the best-case scenario predicting the Australian rabbits will be eliminated after approximately 25 or more generations after deployment (Fig. 4.5). Although migration from Australia is equally unlikely as rabbit migration to Australia, this scenario was included in the modelling. In this scenario, migration would eventually allow gene drives to penetrate Europe, reducing the frequency of the most common haplotype. However, the resistant European haplotype would remain unaffected and increase in frequency (Fig. 4.5A). In a scenario where Europe has a smaller population size of 1000 (1000x smaller than Australia), we would observe the same trends as before, except that gene drive haplotype would infiltrate Europe earlier (Fig. 4.5B). Despite maintaining the same migration rate from Australia in the model, the smaller European population would mean that the influx constituted a larger proportional increase. This disparity would lead to the resistant allele's delayed establishment in Australia, occurring around generation 500, rather than within the original observation period.

Figure 4.5.: Modelling the gene drive spread targeting a region in DNAH17 gene. Blue represents gene drive haplotype; red is the susceptible haplotype with darker colour to black representing increasingly resistant haplotype (A) Equal population size (approximately 1 million) and migration rate at ~ 5 x 10^{-6} (five individual per million individuals per generation); (B) Setting a smaller population size in Europe (approximately 1000) with the same migration rate.



We next investigated the NANOS2 gRNA (mOryCun1.1 scaffold NC_091449.1 at positions 11136815-11136836). There were two haplotypes; the most common was found throughout Australia and was present in approximately 95% in European rabbits, while the other haplotype had a mutation 7 bp upstream of the PAM and was only found in Europe. The conversion efficiency of the second haplotype was approximately 20% (only 20% of pairing of that haplotype with the gene drive haplotype will result in conversion to the gene drive haplotype). Using the same conditions as the models for DNAH17, we also saw that this gRNA target was effective for gene drive spread in rabbits in Australia. However, the partially resistant haplotype in rabbits in Europe would offer minimal resistance to gene drive (Fig. 4.6).

Figure 4.6.: Modelling the gene drive targeting a region in NANOS2 gene. Blue represents gene drive haplotype; red is the susceptible haplotype with darker colour to black representing increasingly resistant haplotype (A) Equal population size (approximately 1 million) and migration rate at ~ 5 x 10^{-6} (five individual per million individuals per generation); (B) Setting a smaller population size in Europe (approximately 1000) with the same migration rate.



In all cases, the locally fixed allele approach was applied to ensure effective gene drive spread within the target population. However, the above examples were specific to homing gene drives. With a perfect homing gene drive, there is always a risk to non-target populations, as it can propagate even from a low frequency, unless resistant haplotypes are present to halt its spread. Conversely, selecting a system where the non-target population is resistant carries a long-term risk of reintroducing the resistant haplotype into the target population, though in our DNAH17 scenario, this process occurred over an extended timeframe.

Other gene drive systems, such as T-CRISPR, daisy-chain gene drives, multi-target gene drives, and hypothetical local drives, function differently. Some require a minimum threshold frequency before spreading, making them more localised.

A more precise evaluation of model outcomes would require additional data on gene function and proof of concept for engineering rabbits with gene drives. The specific impacts of gene drive systems could also be influenced by factors not accounted for in this study, such as variations in gene function and the type of gene drive employed.

4.5 Conclusion – Long term biocontrol pipeline

Our study identified a list of potential gene drive targets and established a generalised framework for modelling the progression of gene drives. The examples presented indicate that the gene drives could be theoretically possible, though the practical implementation will depend on technical feasibility and the strength of their effects on the target genes. The insights gained from this research will guide future work towards developing an additional rabbit biocontrol strategy that may provide an additional tool to reduce the on-going cost to the industry from the damages inflicted by rabbit populations in Australia.

The next steps will depend on demonstrating the feasibility of engineered gene drives. A comprehensive evaluation of gene disruption effects (phenotypes) will be necessary to refine predictions and guide the next phases toward practical application for rabbit population management.

4.5.1 Key findings

- Whole genome sequencing of diverse rabbit samples from Australia in comparison with European data sets led to the detection of an outlier population in South Australia
- We could identity locally fixed alleles in the Australian rabbit population compared to the European population
- We identified two target genes (DNAH17 and NANOS2) that warrant further investigation for a sex-biasing gene drive approach
- Modelling shows that under current assumptions a gene drive is possible and would lead to a population crash in the target population
- More information on gene drive mechanisms, gene function and rabbit movement are required for more accurate modelling

4.5.2 Benefits to industry

This project component showed that we were able to identify suitable genetic signatures in genes related to reproduction and sex determination that could potentially be used as a future gene drive targets for Australian rabbits. This lays the groundwork for any future genetic biocontrol in Australian rabbits. Furthermore, the modelling of these two genes in the context of Australian and European rabbit populations show that an eradication of Australian rabbits without significant effects on European populations is in theory feasible and could complement current viral biocontrol strategies.

5. Scavenger analysis

5.1 Background – Scavenger analysis

With the emergence of RHDV2 in Australia and worldwide, questions have been raised about its host specificity. Those questions arose, because in contrast to RHDV1, RHDV2 shows an expanded host range in lagomorphs, i.e., the virus and can not only infect European rabbits but also hare and cottontail species around the world (Puggioni et al., 2013; Camarda et al., 2014; Neimanis et al., 2018; Kennedy et al., 2021; Asin et al., 2022). This does not only make the virus a threat to native lagomorph species outside of Australia but also sparks concerns about the infection of native animals and introduced scavengers in Australia. Questionable studies have been published over the years that claim infection of badgers and musk deer with RHDV1 and RHDV2 (Bao et al., 2020; dos Santos et al., 2022). Other studies reported trace amounts of RHDV2 RNA in pine voles and white-toothed shews (Calvete et al., 2019). In Australia, metagenomic studies of Tasmanian devils and foxes have identified RHDV RNA in the faeces of those animals (Campbell et al., 2020; Chong et al., 2019). This raises the questions whether scavengers can get infected with RHDV when scavenging on rabbits and if the emerging of a new RHDV genotype poses a threat to other animals.

At the start of this project in 2020, CISS was investigating the merit of the registration of RHDV2 as additional registered biocide. This project aspect aimed at providing critical safety data supporting this registration. In 2022, based on the data generated through the CISS projects the decision was made to not proceed with the registration. At the time, RHDV2 was found to be widespread and cause frequent outbreaks in the populations studied, resulting in high levels of population immunity and few anticipated benefits from the augmentative application of an RHDV2 product.

5.2 Objectives – Scavenger analysis

The objective of this project component was to opportunistically sample scavengers of rabbits (e.g. feral cats, foxes, wild boar). Samples were analysed for the presence of RHDV RNA and antibodies in scavengers to assess exposure to the virus and create materials for potential follow up analysis if results suggested any evidence of infection. Finally, results of this study were published.

- Opportunistically sample scavengers of rabbits
- Test samples for the presence of RHDV RNA and antibodies against the virus
- Establish virus species specificity using organoid infection studies
- Publication of results

All objectives of this project component have been met successfully.

5.3 Methodology – Scavenger analysis

We utilised our network of private shooters, local land services and park rangers in New South Wales, Australian Capital Territory, Queensland and Western Australia to opportunistically collect fresh blood and tissue samples from scavengers, including cats, dogs, foxes and feral pigs.

A RHDV-specific RT-qPCR assay was used to detect viral RNA in liver samples and an in-house RHDV2-specific blocking ELISA was used to determine antibody levels in collected blood samples. Where positive results were achieved, histology was carried out on paraformaldehyde (PFA)-fixed livers to investigate for signs of disease. To test for species specificity, we established organoid

systems for fox, cat and mouse as described above and observed if infection with RHDV2 was possible.

5.4 Results – Scavenger analysis

We were able to collect samples from 99 feral animals. This included 38 foxes, seven cats, eight dogs and 46 pigs. Out of these samples, five samples (two foxes, three cats and one pig) showed only detectable levels of RHDV2 antibodies. An additional two samples (one fox and one cat) showed antibody levels, and we could detect RNA in the faeces. Two foxes showed viral RNA in the faeces but without detection of antibodies. No viral RNA was detected in any of the liver samples. No signs of infection were found in PFA-fixed liver samples via histology of seropositive samples. Results are summarized in Table 5.1

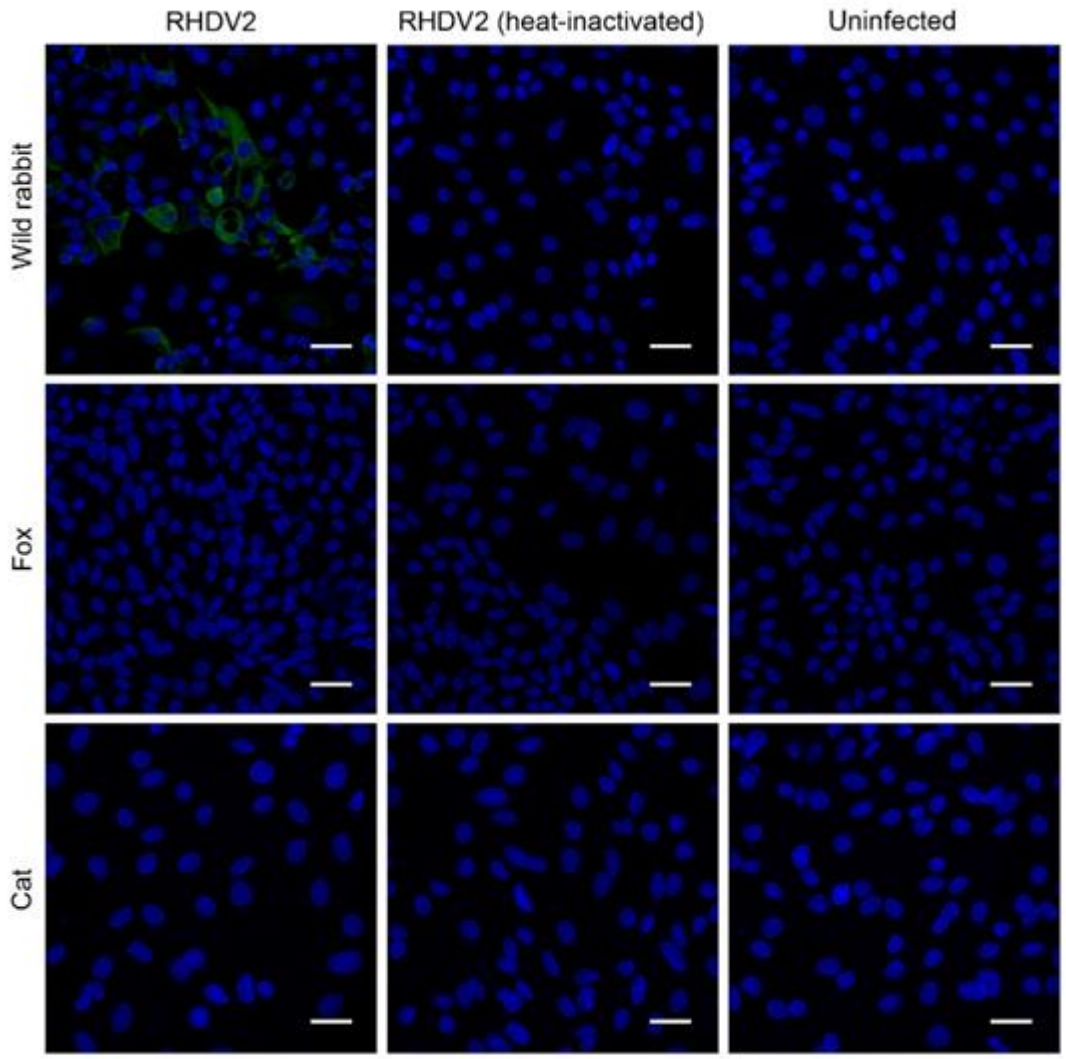
Table 5.1: Evidence of RHDV2 exposure in scavengers, October 2020 to February 2023.

Group	Evidence of RHDV2 exposure			Species			
	Sero-positive	Viral RNA in faeces	Viral RNA in liver	Fox	Cat	Dog	Pig
No exposure detected	-	-	-	33	3	8	45
Antibodies only	+	-	-	2	3	0	1
Faecal RNA only	-	+	-	2	0	0	0
Antibodies + faecal RNA	+	+	-	1	1	0	0
Liver RNA	-	-	+	0	0	0	0
TOTAL				38	7	8	46

To confirm the species specificity, we established organoid cell culture systems of fox and cat and compared RHDV2 infectivity with those of rabbits. Cells were inoculated with RHDV2, heat-inactivated RHDV2 and mock inoculated with cell culture medium. Infectivity could only be shown for cells derived from rabbits and hares (Fig. 5.1), in line with the species specificity observed for RHDV2.

The results of this study have been published in the journal *Wildlife Research* (Smith et al., 2025).

Figure 5.1: Rabbit, fox, and cat hepatobiliary cells 24 hours after inoculation. Monolayer cultures derived from rabbit, fox and cat hepatobiliary organoids were grown in chamber slides and inoculated with 100 RID_{50} of RHDV2. Cultures were fixed, permeabilized and immunostained at 24 hpi using a mouse mAb directed against the RdRp of RHDV2 (green); DNA was stained with DAPI (blue). The control groups consisted of cells mock inoculated with culture medium (right column), and another group exposed to the same titre of heat-inactivated RHDV2, achieved by heating at 63°C for 10 minutes in a water bath (middle column) Scale bar, 50 µm.



5.5 Conclusion – Scavenger analysis

Our results show that RHDV2 RNA can be detected in faeces and liver samples of rabbit scavengers and predators and that animals can also develop an immune response with detectable antibody levels against RHDV2. However, pathology and histology of liver samples from those animals show no sign of infection and no antigen could be detected. To further strengthen those findings, we developed liver organoid cell culture systems for cats and foxes. No infection was observed in cat and fox liver organoids. It can therefore be concluded that there is a negligible risk of RHDV2

infection in scavengers and predators. However, they play an important role in spreading the virus through their faeces.

5.5.1 Key findings

- Detection of RHDV2 RNA and antibodies in pigs, foxes and cats
- No signs of infection in pathology and histology
- No infection of liver organoids derived from cats and foxes
- RHDV2 is lagomorph species specific

5.5.2 Benefits to industry

The results show that RHDV2 poses no risk to species beyond the order Lagomorpha. Species specificity could furthermore be confirmed using organoid cell culture systems. Should the registration of RHDV2 as an augmentative biocide be revisited in the future, the outputs from this project provide assurance that it will pose no risk to species other than rabbits and hares, even if they are exposed to very high concentrations of virus. With the use of organoid cell culture systems to confirm species specificity we also lay the groundwork to ensure species specificity for RHDV variants that will be developed in the future using accelerated evolution under antibody pressure as described above.

6. Economic Assessment

6.1 Background – Economic Assessment

The final year of the project also included an economic analysis of the likely benefits if the project outputs were adopted (this report). Project P.PSH.1059 included several lines of research aimed at the potential development or identification of new biocontrols, e.g., developing a culture system for RHDVs to facilitate future natural selection of additional variants as biocontrol tools (Section 1), the search for new rabbit pathogens (bioprospecting, Section 3) and investigating the feasibility of novel gene drive technologies for rabbit control in Australia (Section 4).

While all sub-projects delivered on their associated milestones, no strong candidate for a new suitable rabbit pathogen was identified through the bioprospecting approach. Gene drive technology was found to be theoretically feasible for future rabbit control in Australia based on population genomics analysis; however, proof-of-concept has yet to be demonstrated in rabbits and parameters such as development costs and timelines are highly uncertain at this time. By contrast, the RHD-Accelerator sub-project successfully delivered a robust cell culture system, paving the way for the selection of new RHDV variants for potential future registration and release. The RHD-Accelerator approach of accelerating natural selection is repeatable, and work on the subsequent variant can commence as soon as a new variant is released, enabling a more pro-active delivery of the rabbit biocontrol pipeline and removing Australia's dependency on the serendipitous emergence of new virulent rabbit pathogens every 10-15 years. Furthermore, as an RHDV-based biocontrol, a wealth of data is available for the development and impacts of previous registered biocontrols to inform the input parameters for the economic assessment. Consequently, the RHD-Accelerator approach was selected as the project output for an in-depth economic analysis, which will be used to strengthen the business case for future investment into a sustainable rabbit biocontrol pipeline.

6.2 Objectives – Economic Assessment

The objective of the project was to estimate the likely cost-benefit ratios if the proposed biocontrol pipeline, in this case the RHD-Accelerator platform technology, was implemented. The objectives were met, and an in-depth analysis is provided as a separate report (Azeem et al., 2025).

6.3 Methodology – Economic Assessment

The economic analysis builds on previous economic assessments of RHDV impacts (Hardaker et al., 2020), but with modifications where new information was available to better inform modelling parameters. We used Net Present Value (NPV) and the Benefit-Cost Ratio (BCR) as financial metrics to evaluate the economic viability of putative new RHDV variants. The NPV incorporates the time value of money by comparing the present value of expected benefits to the cost of investment. To enable meaningful comparisons of benefits and costs over time, it was necessary to account for inflation, as the value of money diminishes over time. Rabbit impact and control costs were adjusted to 2024-dollar terms using the Implicit Price Deflator for Gross Domestic Product (GDP) provided by the Australian Bureau of Statistics (Australian Bureau of Statistics, 2024). Development costs and timelines were informed by previous research projects aimed at assessment, registration and rollout of RHDV-based biocontrols and biocides, namely RHDV1-K5 and RHDV2, and adjusted to 2024 AUD terms. The resulting adjusted total development costs were estimated at $27,3 million over 13 years

(starting in 2026), with a virus release in 2036 and a subsequent two-year impact monitoring period. A discount rate of 7% was applied for initial analyses in line with recommendations (Hone et al., 2022). We used available data from previous RHDV-based biocontrols to guide the modelling of two types of scenarios we considered likely to provide the best and worst-case impact scenario (Ramsey et al., 2020), with the expectation that the impact of any new virus produced through the RHD-Accelerator project would sit within these boundaries. The best-case scenario here is a virus with properties similar to RHDV2, but species specific to rabbits, that would spread amongst rabbit populations leading to an average knock down of 60%, in 100% of the areas inhabited by rabbits (case study 1). A lower impact case scenario was informed by RHDV-K5, a Korean strain of RHDV that was released nationwide in 2017 which did not spread significantly beyond release sites and acted more like a local biocide, affecting 5% of areas affected by rabbits (case study 2). For both scenarios we modelled a one-off release, as well as three repeat scenarios, with releases in 2036, 2046 and 2056. Several risk factors were considered, including the socio-political risk of project outputs not being approved for release due to insufficient levels of public acceptability.

The two main financial metrics used in this report were the Net Present Value (NPV) and the Benefit-Cost Ratio (BCR) for various possible project outputs over a timeframe of 40 years (2024 – 2064). A summary and range of input parameters for the two case studies is outlined in Table 6.1 below. Sensitivity analyses were carried out using a low (3%) and high (10%) discount rate, as well as selected relevant scenarios varying key parameters such as socio-political risk as well as varying abilities to reduce rabbit costs (as a function of rabbit populations).

Table 6.1: Summary of input data and assumptions sued in the evaluation of RHD-Accelerator output impacts.

Counterfactual variables	**Assumption/ value**	**Source/notes**
Average annual costs of rabbits to industry	$131 m/year	Hafi et al, adjusted to 2024$ terms (Hafi A et al., 2023)
Average annual costs of rabbit control to industry	$94 m/year	Hafi et al, adjusted to 2024$ terms (Hafi A et al., 2023)
Anticipated increase in rabbit impact and control costs over time (p.a.)	3%/year	Guided by/modified from (Hardaker et al., 2020)
Maximum cap on rabbit increase/rabbit impact and control costs	350% of 2022 levels	Guided by/modified from (Hardaker et al., 2020)
Implementation of pipeline		
Accelerator project output variables	**Assumption/ value**	**Source/comments**
Anticipate reduction of annual rabbit impact costs	30-60%	Based on RHDV-K5 and RHDV2 impacts (Hardaker et al., 2020; D. S. L. Ramsey et al., 2020)
Anticipated reduction in annual rabbit control costs	30-60%	Based on RHDV-K5 and RHDV2 impacts (Hardaker et al., 2020; D. S. L. Ramsey et al., 2020)
Area inhabited by rabbits that will benefit	5%-100%	Based on RHDV-K5 and RHDV2 impacts (Hardaker et al., 2020)
Year of first release	2036	Estimate based on development/registration time for RHDV-K5
First year of realised impact	2037	Assuming a coordinated national release in 2036
Duration of impact	8 years post release	Based on RHDV-2 suppression data (conservative estimate) (D. S. Ramsey et al., 2023)
Timeframe for estimated benefits	40 years	Years 2025 – 2064, allows modelling of 2 repeat scenarios (total of 3 releases)
Anticipated increase in rabbit impact (=rabbit numbers) after eight-year impact period	3% /year	Same as counterfactual
Maximum cap on rabbit increase/rabbit impact and control costs	350% of 2022 levels	Same as counterfactual
Risk factors and discount rates	**Assumption/ value**	
Discount rate	7%	3%, 7% and 10% for sensitivity analyses
Technical risk (probability of output failure)	10%	This risk was ranked low as the methods applied have been used successfully for a range of other viruses in the past (e.g. (Andreano et al., 2021)).
Socio-political risk (probability of no release approval)	Range: 10% - 70%	An important risk that captures possible lack of public acceptance due to a 'man-made' virus. This risk was estimated to be proportional to the ability of a new virus variant to self-spread.
Risk of no usage (if approved, registered and available)	1%	This risk was considered extremely low based on previous experience of uptake of available biocontrol products.
Risk of lack of sustained funding	5%	Moderate risk.

6.4 Results – Economic Assessment

The analysis found that case study 1, the best-case scenario of a self-spreading new RHDV variant with a 60% knock down resulted in a NPV of $1,570 million (BCR 96.5:1) over 40 years with one release in 2036, and $1,826 million and $1,852 million (BCR 74.7:1 and 64.8:1) after repeat releases two and three in 2046 and 2056, respectively (Table 6.1, row

2). Sensitivity analyses including varying discount rates, reduced rabbit knock down rates and socio-political risk ratings all revealed very high returns on investment (Table 6.1, rows 1-9).

For example, the socio-political risk of releasing a new self-disseminating RHDV variant that can overcome immunity to existing strains may be higher, even if the new variant was species-specific to rabbits only and a vaccine was available for pet rabbits. Rows 4-5 (Table 5) illustrate how increasing this risk to 70% (reducing the overall likelihood of success to 25%) affects the risk adjusted NPVs and BCRs. At a 7% discount rate, the risk adjusted NPV is reduced from $924 million to $386 million for the first release and $445 million and $449 million for the second and third release (from $1,073 million and $1086 million, respectively). While reduced, the BCRs of 24.5:1, 19:1 and 16.5:1 for the one- two- and three release scenarios illustrate the potential high value of the investment even when very high risk penalties are applied.

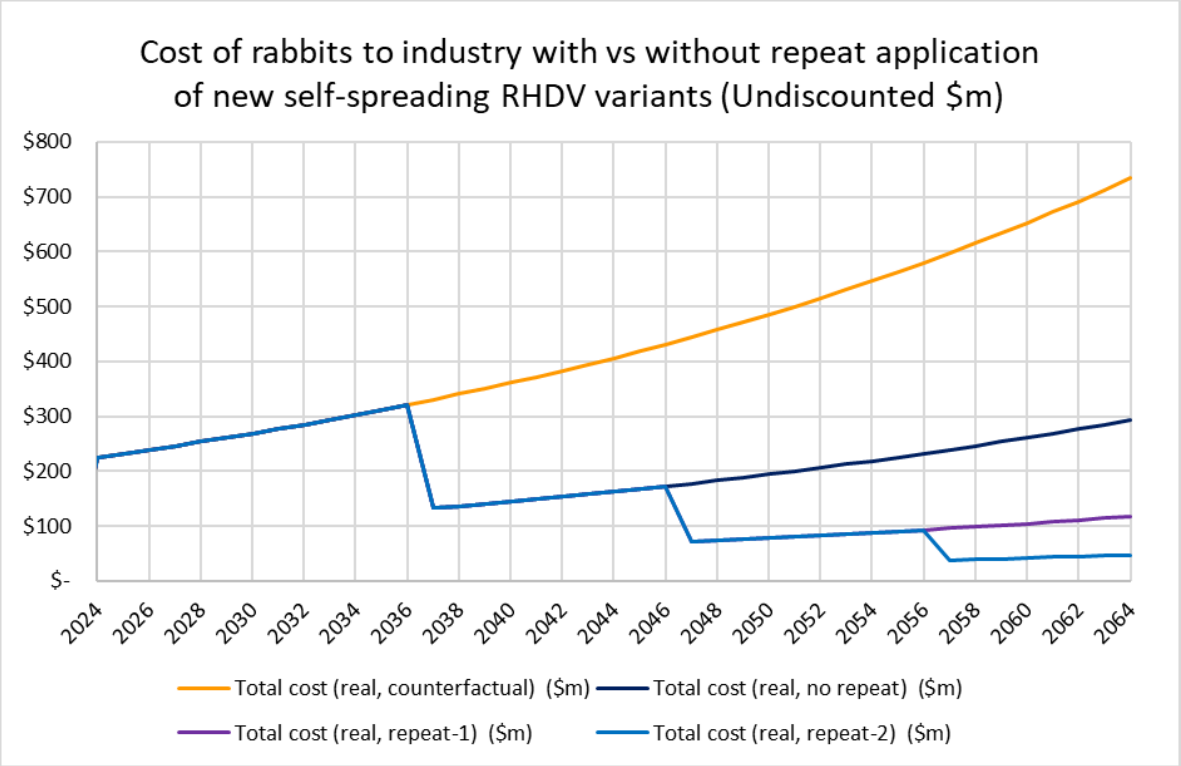
Lastly, we included a scenario into the sensitivity analyses that includes a hypothetical self-spreading new biocontrol agent that achieves a reduced overall lower knock down rate of 30% (and consequently 30% cost reduction rate) on average, while retaining the high socio-political risk parameter of 70%. Rows 7-9 (Table 5) show that even with the reduced rabbit impact reduction and low overall probability of success due to the high risk penalties, the NPV is still positive at $230 million after the first release; and $293 million and $302 million after the second and third release respectively, with favourable BCRs of 16:1, 12.8 and 11.4, respectively.

Table 6.1: Sensitivity analyse for case study 1: Self-spreading biocontrol agent. SA = sensitivity analysis, DR = discount rate, NPV = net present benefit, BCR = benefit cost ratio, RA = risk adjusted. Default discount rate scenarios (7%) are in bold. Yellow highlights indicate key changes in parameters for the sensitivity analyses.

SA	DR	Cost reduction	Area	Socio political risk	overall likelihood of success	Release 1 NPV	Release 1 BCR	Release 2 NPV	Release 2 BCR	Release 3 NPV	Release 3 BCR	Release 1 NPV-RA	Release 1 BCR-RA	Release 2 NPV-RA	Release 2 BCR-RA	Release 3 NPV-RA	Release 3 BCR-RA
1	3%	60%	100%	30%	59%	$ 3,759	173.8	$ 4,715	125.3	$ 4,875	98.5	$ 2,218	103.0	$ 2,778	74.2	$ 2,868	58.4
2	**7%**	**60%**	**100%**	**30%**	**59%**	**$ 1,427**	**87.8**	**$ 1,717**	**70.3**	**$ 1,756**	**64.5**	**$ 839**	**52.0**	**$ 1,007**	**41.6**	**$ 1,029**	**36.4**
3	10%	60%	100%	30%	59%	$ 746	56.2	$ 870	47.5	$ 884	43.6	$ 436	33.3	$ 508	28.1	$ 515	25.8
4	3%	60%	100%	70%	25%	$ 3,759	173.8	$ 4,715	125.3	$ 4,875	98.5	$ 938	44.1	$ 1,169	31.8	$ 1,201	25.0
5	**7%**	**60%**	**100%**	**70%**	**25%**	**$ 1,427**	**87.8**	**$ 1,717**	**70.3**	**$ 1,756**	**64.5**	**$ 350**	**22.3**	**$ 418**	**17.8**	**$ 424**	**15.6**
6	10%	60%	100%	70%	25%	$ 746	56.2	$ 870	47.5	$ 884	43.6	$ 179	14.3	$ 207	12.1	$ 209	11.1
7	3%	30%	100%	70%	25%	$ 1,868	86.9	$ 2,703	72.2	$ 2,955	60.1	$ 458	22.1	$ 658	18.3	$ 713	15.3
8	**7%**	**30%**	**100%**	**70%**	**25%**	**$ 705**	**43.9**	**$ 958**	**39.7**	**$ 1,020**	**36.1**	**$ 167**	**11.2**	**$ 225**	**10.1**	**$ 237**	**9.2**
9	10%	30%	100%	70%	25%	$ 366	28.1	$ 474	26.3	$ 497	24.9	$ 83	7.1	$ 107	6.7	$ 111	6.3

It is worth noting that this scenario also illustrated how repeated releases of such putative new self-spreading viruses before rabbit populations recovered were able to drive down rabbit costs to industry (equivalent to a reduction in rabbit numbers) to ~10% of 2022 levels (Figure 6.1). While economic returns on investments diminish with every repeat virus release as rabbit impacts are reduced from a lower basis, this strategy would result in substantial environmental benefits due to rabbit numbers gradually declining over the 40-year period towards levels not observed since the release of RHDV1 in 1996.

Figure 6.1: Total real future costs of rabbits to industry without and with up to three releases of putative new self-spreading RHDV variants (case study 1), from 2024, with virus releases in 2036, 2046 and 2056.



Case study 2, a non-spreading biocide yielded an estimated NPV of $74m (BCR5.5:1) over 40 years with one release, and $110 million and $120 million (BCR 5.5:1 and 5.2:1) after repeat releases two and three, respectively (Table 6.2, row 11).

A hypothetical scenario of an intermediate putative virus variant with a 40% knock down rate and some ability to spread between populations was included into the sensitivity analyses as an example for a putative project output with an anticipated impact between the two extreme scenarios (row 13-15). We further varied parameters in this sensitivity analyses to model a putative new virus variant with traits that places it between the two extreme case study scenarios. Row 16-18 (Table 6.2, rows 19-21) shows the calculations for a putative virus that would achieve a moderate knock down of 40% but exhibit some limited spread over ~25% of the areas affected by rabbits, which raises the NPV to $309 million for the first release ($232 million risk adjusted, r0ws 16-18). This example illustrates the sharp increase in value if a virus variant has capacity to spread beyond release sites. Even if such a virus was penalised with increased (30%) socio-political risks reducing

the overall likelihood of success to 59% (Rows 19-21), the risk adjusted NPV was still $177 million for the first and $252 million for two and $271 million for three consecutive releases, with a BCR of 11.8:1 and 11.2:1 and 10.3:1, respectively, at a 7% discount rate.

We carried out further sensitivity analyses varying different parameters, to test the minimum efficacy any putative product would need to have to remain financially viable. Rows 22-27 assume low rates of uptake and almost no spread beyond release sites, reducing the treated to 2% (Rows 22-24) and 1% (Rows 25-27), respectively. This scenario was informed by assumptions made in a previous economic assessment (Hardaker 2022), that assumed a treated area of 1.8% of land inhabited by rabbits in the first year, and 0.8% in subsequent years. For the 2% treated are scenario, the NPVs and BCRs remain positive for all three discount rates, although at the higher discount rates and with risk adjustment, the model no longer predicts positive returns on investments. Similar results were obtained for a scenario where a putative control agent would only reduce populations in 2% treated areas by 20% (Rows 28-30). A virus with such properties would not be considered a suitable candidate for registration.

As outlined above, the RHD-Accelerator method has the potential to deliver both new self-spreading biocontrols as well as improved local biocides. Although not included into the scenario modelling, registration and application of several such viruses in combinations is theoretically feasible and could further improve efficacy of applications and a further increase in returns on investment. For example, RHDV-based biocides that can partially overcome immunity to circulating strains could be used to further reduce rabbit populations following natural outbreaks. If several such biocides were available, these could be used in rotation to maximise the removal of immune survivors of natural outbreaks.

Table 6.2: Sensitivity analyse for case study 2: New biocide. SA = sensitivity analysis, NPV = net present benefit, BCR = benefit cost ratio, RA = risk adjusted.

SA	Discount rate	Cost / impact reduction	Area	Socio political risk	overall likelihood of success	Release 1 NPV	Release 1 BCR	Release 2 NPV	Release 2 BCR	Release 3 NPV	Release 3 BCR	Release 1 NPV-RA	Release 1 BCR-RA	Release 2 NPV-RA	Release 2 BCR-RA	Release 3 NPV-RA	Release 3 BCR-RA
10	3%	60%	5%	10%	76%	$ 167	8.7	$ 269	8.7	$ 308	7.2	$ 122	6.6	$ 196	6.2	$ 223	5.5
11	**7%**	**60%**	**5%**	**10%**	**76%**	**$ 56**	**4.4**	**$ 84**	**4.4**	**$ 92**	**4.2**	**$ 39**	**3.3**	**$ 58**	**3.3**	**$ 63**	**3.2**
12	10%	60%	5%	10%	76%	$ 24	2.8	$ 35	2.9	$ 38	2.8	**$ 15**	2.1	$ 22	2.2	$ 24	2.1
13	3%	40%	5%	10%	76%	$ 104	5.8	$ 167	5.4	$ 190	4.8	$ 74	4.4	$ 119	4.1	$ 133	3.7
14	**7%**	**40%**	**5%**	**10%**	**76%**	**$ 32**	**2.9**	**$ 48**	**2.9**	**$ 52**	**2.8**	**$ 20**	**2.2**	**$ 30**	**2.2**	**$ 33**	**2.1**
15	10%	40%	5%	10%	76%	**$ 12**	1.9	$ 17	1.9	$ 18	1.9	**$ 6**	1.4	**$ 9**	1.5	**$ 9**	1.4
16	3%	40%	25%	10%	76%	$ 608	29.0	$ 957	26.2	$ 1,090	22.8	$ 458	22.1	$ 720	20.0	$ 819	17.4
17	**7%**	**40%**	**25%**	**10%**	**76%**	**$ 224**	**14.6**	**$ 328**	**14.2**	**$ 360**	**13.4**	**$ 167**	**11.2**	**$ 244**	**10.8**	**$ 267**	**10.2**
18	10%	40%	25%	10%	76%	$ 113	9.4	$ 156	9.4	$ 168	9.1	$ 83	7.1	$ 115	7.1	$ 123	6.9
19	3%	40%	25%	30%	59%	$ 608	29.0	$ 957	26.2	$ 1,090	22.8	$ 352	17.2	$ 551	15.5	$ 626	13.5
20	**7%**	**40%**	**25%**	**30%**	**59%**	**$ 224**	**14.6**	**$ 328**	**14.2**	**$ 360**	**13.4**	**$ 126**	**8.7**	**$ 184**	**8.4**	**$ 201**	**7.9**
21	10%	40%	25%	30%	59%	$ 113	9.4	$ 156	9.4	$ 168	9.1	$ 61	5.5	$ 85	5.5	$ 91	5.4

6.5 Conclusion – Economic Assessment

The economic assessment reveals substantial positive returns on investment following the implementation and translation of the putative project outputs for rabbit biocontrol in Australia. NPVs and BCRs increase significantly with the ability of a putative new virus strain to self-disseminate and spread amongst rabbit populations, with anticipated impacts similar to those seen following the arrival of RHDV2 in Australia. NPVs and BCRs for local biocides are greatly reduced but still positive.

The likely repertoire of possible viruses could range from a self-spreading biocontrol virus with impacts comparable to RHDV2 to improved local biocides that have limited ability to spread but can be used locally to improve integrated rabbit management outcomes by partially overcoming immunity to circulating RHDVs. Although not included in the modelling, combined applications of several RHD-Accelerator variants are theoretically possible and could further increase the efficacy of rabbit control operations.

While the RHD-Accelerator method uses natural processes of selection and not genetic modification, the resulting viruses are still 'man-made' and the associated contentiousness may lead to increased socio-political risks impeding registration and national release. We accounted for this in the sensitivity analysis by increasing the socio-political risks which still resulted in high NPVs and BCRs, highlighting the high potential value of any investments into this approach even in the face of high-risk penalties.

Taken together, the results presented here make a strong case for continued investment into the RHD-Accelerator approach as part of the long-term rabbit biocontrol pipeline that is likely to result in high returns on investment.

6.5.1 Key findings

- Ongoing investment into RHDV-based rabbit biocontrol agents is economically justified
- The impacts and returns on investment are greater for a self-spreading biocontrol agent compared to a non-spreading local biocide, although the latter still returns positive BCRs.
- If successful, the repeatable nature of the approach could potentially lead to a gradual reduction of rabbit levels and impacts not seen since the release of the first RHDV in 1996, if new variants were available and applied before populations had an opportunity to fully recover from the impact of the previous biocontrol.
- The RHD-Accelerator method has the potential to produce new self-spreading biocontrols as well as additional biocides that could be used standalone or in combination.

6.5.2 Benefits to industry

The economic analysis provides a valuable tool to guide investment decisions into sustainable long-term reduction of rabbit impacts to industry and the environment. Successful implementation of the RHD-Accelerator pipeline can be expected to deliver substantial, long-lasting benefits to industry with potentially very high returns on investments.

7. Future research and recommendations

Project P.PSH.1059 has provided a strong foundation for developing innovative biocontrol solutions to manage Australia's most destructive vertebrate pest. Future research building on the project's outputs will achieve continued progress in mitigating the agricultural damage caused by invasive rabbit populations, as effective rabbit biocontrol translates to improved pasture availability, increased agricultural output, reduced land degradation and lower rabbit management costs.

Three project areas of P.PSH.1059 warrant future research: (1) the RHD-Accelerator, (2) emerging genetic technologies for pest animal population control (3) bioprospecting.

7.1. Continue RHD-Accelerator technology for the development of new virus variants for future releases.

The organoid sub-project successfully developed the first robust cell culture system for propagating RHDVs, the first in 40 years of world-wide research. The culture system was further optimised to allow for the successful passaging of RHDV in cultured cells. The successful completion of this project aspect is now paving the way for commencing the selection of superior RHDV-based biocontrol tools for subsequent release by accelerating natural selection processes. The project was also able to demonstrate that organoid cultures can be used to study species specificity of various RHDVs, which is a critical safety requirement for any viral biocontrol. The ability to replicate (or not) in liver organoids derived from rabbits, hares, cats, foxes and mice accurately reflected the species-specificity of the viruses in the natural hosts.

The economic assessment of the RHD-Accelerator implementation presents a strong business case for future investment in this approach, with potentially very high returns on investments and long-term benefits to Industry through the sustainable reduction in rabbit numbers and impacts. Based on previous RHDV-based rabbit biocontrols, the estimated timeframe for development is 10 years.

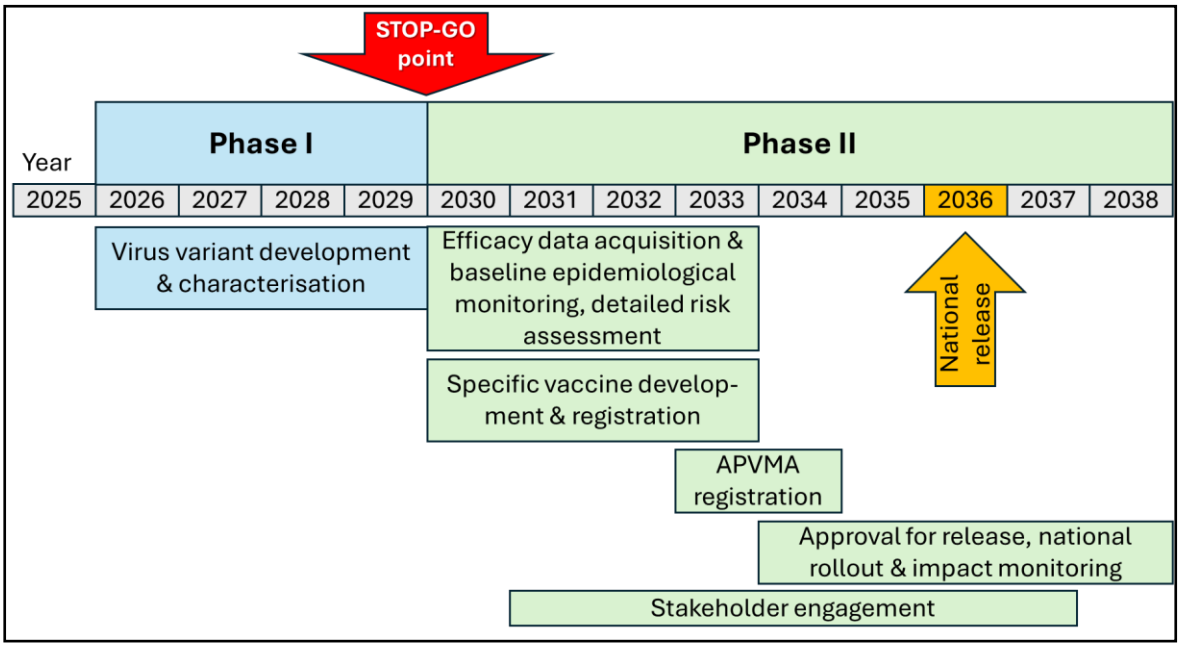
Since the arrival of RHDV2 in Australia in 2014, rabbit populations have been at historically low levels. However, based on experience with previous biocontrols, its impact is likely to wane, and rabbit numbers may soon start to increase again. Continuing the RHD-Accelerator approach and working towards the next rabbit biocontrol virus and removing Australia's dependence from the serendipitous emergence of new virulent rabbit diseases is therefore a priority.

Going forward, the RHD-Accelerator approach can broadly be divided into two phases (Figure 7.1):

Phase I: production of new RHDV-based rabbit biocontrol virus candidates

Phase I will deliver proof-of-concept that the RHD-Accelerator platform technology can be used to produce a series of candidate virus variants that can overcome immunity against circulating viruses, while retaining their virulence and species specificity. This involves the passaging of RHDVs in culture systems developed through this project, under the selection pressure of neutralising antibodies. Basic species specificity profiles can also be determined in liver organoids derived from rabbits, hares, and non-target species such as cats, foxes or mice to ensure that any candidate virus poses no danger to lagomorph species other than rabbits. However, animal infection studies will be required to assess the ability of the candidate viruses to fatally infect very young rabbits (a key epidemiological advantage), and to prevent a loss of virulence resulting from adaptation of the virus to cell culture conditions. Building on the successful outputs from project P.PSH.1059, Phase I can commence now.

Figure 7.1: The two implementation phases of the RHD-Accelerator platform technology. APVMA = Australian Pesticides and Veterinary Medicines Authority.



Phase II: Registration, release and impact monitoring

Pending successful delivery of Phase I and generation of suitable variants with acceptable efficacy and risk potential, Phase II can proceed towards the registration and national release of the next next RHDV-based biocontrols. The broad work packages likely required for Phase II are outlined in Figure 7.1 and are informed by the assessment and/or registration processes for RHDV1-K5.

Efficacy data acquisition, baseline epidemiological monitoring and APVMA registration:

In preparation for registration with the APVMA, larger-scale efficacy testing of the candidate viruses in will likely be required. This may include (but is not limited to) virulence testing in domestic and wild rabbits, quantifying the degree to which it can overcome immunity to circulating viruses, and possibly limited non-target species testing in animals. In parallel, field epidemiological studies need to ascertain the current level of population immunity to various circulating strains as well as rabbit abundance, to inform the development of national release strategies and the estimated impacts.

Detailed risk assessment and stakeholder engagement:

Any effective biocontrol that will spread and effectively reduce rabbit populations in Australia naturally poses a risk to rabbits in their native range (i.e., the Iberian Peninsula). Previous successful biocontrol agents were all emerging rabbit diseases that first appeared outside Australia and were either deployed as biocontrols (e.g., Myxoma virus and the original RHDV released in the mid-1990s), or they spread without being deliberately released (e.g., RHDV2 in 2014). If viruses produced by the proposed project are considered for registration and release, Australia would for the first time not just be the recipient of an emerging rabbit disease, but the origin of one. This requires a thorough risk assessment weighing the potential environmental and economic benefits against the risks (including global, reputational, trade) and explore mitigation strategies. Early, transparent and ongoing engagement with stakeholders, including the general public, is highly recommended to gauge the levels of acceptability.

Specific Vaccine:

A new antigenic variant that can overcome immunity to circulating RHDVs will also likely overcome protection from the current vaccine. To ensure safety of non-target rabbits such as farmed and pet rabbits, and to help mitigate socio-political risks outlined above, availability of an updated vaccine formulation for owned rabbits well ahead of any proposed releases is highly advisable. While the development of such a vaccine is not necessarily the role of the meat industry, there are possible synergies with other MLA-enabled work, e.g. the development of novel mRNA-based vaccine platform technologies (P.PSH.1444) for the rapid response to emergency animal diseases.

Approval for release, national rollout and impact monitoring:

Any new RHDV-based biocontrol will have to progress through the regulatory and policy pathways mapped out by previous RHDV-based biocontrols (e.g., RHDV1-K5). In addition to registration by the APVMA as a biopesticide product, implementing a national rollout of any new biocontrol will require review in the context of relevant legislation (e.g., the Environmental Protection and Biodiversity Conservation (EPBC) Act and/or the Biocontrol Act), as well as a public consultation period. Ongoing stakeholder and public engagement through this process will be critical.

7.2 Advanced long-term biocontrol pipeline options using genetic control technologies

The project investigated the theoretical possibilities for successful gene drive control of rabbits in Australia. By sequencing the entire genome of over 360 individual wild rabbits from 114 locations across Australia and incorporating genome data from collaborators of an additional 180 wild rabbit and domestic rabbits, we generated an Australian rabbit genome database that is essential for any type of gene drive research going forward in rabbits. Identification of locally fixed alleles in the Australian rabbit population and subsequent modelling of the theoretical spread suggests that an eradication of Australian rabbits without significant effects on European populations is in theory feasible. However, research is still in its early stages, with proof-of-concept for a mammalian gene drive only reported in the mouse model so far (Gierus et al., 2022). In order to adapt this new technology for rabbit control, both theoretical and technical development work needs to be progressed.

Theoretical work could develop more tailored gene drive proof-of-concept models for feral rabbit biocontrol. Additional genetic analyses could refine models and allow incorporation of possible effects of polyandry (multiple paternity) on gene drive spread, or any trade-off effects of the gene drive individuals, e.g., reduced reproductive success or shortened life span. Incorporating these biological factors into gene drive models will help refine predictive models for efficacy and risk assessment.

More urgently needed are technical improvements of genetic control technologies for key pest animals. The gene drive developed in mice utilises a genetic mechanism that has not been identified outside of rodents, therefore the development of a genetic tool that can bias inheritance ratios in other species (including rabbits) is critical. Prerequisite to accelerating research towards gene drives for key pest animals is the development of improved gene editing technologies in non-model animal species, and the rabbit is the next logical step up from the mouse model. Such transferable technologies would have broader applicability and potential benefits to the meat industry beyond

pest control. Some examples are improving livestock genetics for increased disease or parasite resistance, improved temperature tolerance, and sex-biasing technology producing all-male offspring to increase average carcass size.

In summary, while the development of gene drive technology for rabbit population control is likely decades away, future investment into these technologies is warranted, especially if the acceptance for lethal control tools decreases. In addition, technological advances during the development of the technology may have broader applicability and yield returns on investment in other areas.

7.3. Revisit Bioprospecting strategies if RHD-Accelerator proves unsuccessful.

This project component developed a comprehensive framework for detecting pathogens in biological samples and successfully identified a new virus in lagomorphs as well as several known pathogens in unexpected locations. Hepatitis E virus was confirmed in Australian rabbits for the first time, a novel hepacivirus was discovered in North American lagomorph species, and benign caliciviruses were found to be present in Chilean rabbits. None of these viruses were deemed strong candidates to further develop as future biocontrols, especially when compared to the RHD-Accelerator approach that has a much higher likelihood of success. However, should the RHD-Accelerator approach prove to be unfeasible, some of the pathogens uncovered here may warrant further investigation.

Due to its zoonotic potential, HEV is not a suitable biocontrol candidate. However, as it targets the same organ as RHDVs (the liver), it may be worth investigating if it has a possible synergistic effect on RHDV efficacy. Monitoring of HEV activity in wild rabbits may help identify windows of opportunity for targeted RHDV applications with improved outcomes.

The novel hepacivirus was discovered in cottontail rabbit and hare species but was not found to be associated with fatalities in these animals. Viruses of this family (such as the human hepatitis C virus) often cause asymptomatic acute infections that can later turn into persistent infections and late development of liver carcinoma in some patients. This virus was therefore not deemed a strong candidate as a future biocontrol agent, as it is lacking the high virulence of acute infections that account for the success of RHDVs and Myxoma virus, and most wild rabbits are not expected to live long enough for such persistent infections and late complications to have a noticeable effect on populations. However, it is theoretically possible that the hepacivirus could exhibit a different (i.e. higher) level of virulence in a different host, similar to what was observed when Myxoma virus jumped from cottontail rabbits to European rabbits. Small-scale pilot infection studies of European rabbits with the new lagomorph hepacivirus could provide these answers. It needs to be noted in this context that this virus is not endemic to Australia and would therefore be subject to import controls and appropriate biological safety containment facilities for any experimental work. Alternatively, such work could be done outside of Australia in countries where the virus is endemic.

The project also expanded international collaborations and networks with scientists interested in the impacts of invasive rabbits. Maintaining these networks will be critical for continuous horizon scanning for disease outbreaks and mortality in rabbits and other lagomorphs worldwide, to identify potential future biocontrol agents. The new benign rabbit calicivirus was discovered in Chile is also relevant in this context. Virulent RHDVs are believed to have evolved from such benign caliciviruses multiple times in both rabbits and hares, so keeping a watching brief on rabbit populations known to carry these progenitor viruses is warranted.

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9. Appendix

9.1 Publications resulting from the project (cumulative)

Type (e.g., ISI journal paper, book chapter, etc)	Authors and title	Journal	Status	Doi/link (once published)
ISI journal paper	Kardia E, Frese M, Smertina E. Strive T, Sheng XL, Estes M and Hall RN. **Culture and differentiation of rabbit intestinal organoids and organoid-derived cell monolayers** **This paper made the top 100 downloaded papers in the "cell and molecular biology category" for *Scientific Reports* in 2021** (2,992 article downloads in 2021)	*Nature Scientific Reports*	Published 08/03/2021	https://doi.org/10.1038/s41598-021-84774-w
ISI journal paper	Jenckel, M, Hall, RN and Strive T: **First description of Hepatitis E virus in Australian rabbits**	*Australian Veterinary Journal*	Published 26/4/2021	https://doi.org/10.1101/2020.10.05.327353
ISI journal paper	Jenckel, M., Smith, I., King T., West P., Taggart PL., Strive T., Hall RN., **Distribution and Genetic Diversity of Hepatitis E Virus in Wild and Domestic Rabbits in Australia** This paper was a feature paper for the special issue.	*Pathogens*, Special Issue "Hepatitis E and the One-Health Aspect: A Threat to Mankind and Animality?"	Published 17/12/21	https://doi.org/10.3390/pathogens10121637
ISI journal paper	Jenckel, M., Hall RN., Strive T., **Pathogen profiling of Australian rabbits by metatranscriptomic sequencing.**	*Transboundary and Emerging Diseases*	Published 22/06/2022	https://doi.org/10.1111/tbed.14609

ISI journal paper	Kardia E*, Fakhri O*, Pavy M*, Mason H, Huang N, Smertina E, Estes M, Strive T, Frese M, and Hall RN., **Hepatobiliary organoids derived from leporids support the replication of hepatotropic lagoviruses.**	*Journal of General Virology*	Published 16/08/2023	https://doi.org/10.1101/2022.04.07.487566
ISI journal paper	Smertina E, Keller LM, Huang N, Flores-Benner G, Correa-Cuadros JP, Duclos M, Jaksic FM, Briceño C, Neira Ramirez V, Díaz-Gacitúa M, Carrasco-Fernández S, Smith IL, Strive T and Jenckel M., **First Detection of Benign Rabbit Caliciviruses in Chile.**	*Viruses*, Special Issue "Rabbit Viral Diseases"	Published 12/03/2024	https://doi.org/10.3390/v16030439
ISI Journal paper	Ina L. Smith, Nina Huang, Megan Pavy, Alexander Gofton, Omid Fahri, Egi Kardia A, Roslyn Mourant, Sammi Chong, Maria Jenckel, Robyn N. Hall and Tanja Strive **No evidence of rabbit haemorrhagic disease virus 2 infection in scavengers of wild rabbits in Australia.**	*Wildlife Research*	Published 14/01/2025	https://doi.org/10.1071/WR24122
ISI Journal paper	Maria Jenckel, Wei-Shan Chang, Emily A. Wright, Robert Bradley, Robert J. Dusek, Hon S. Ip, Robyn N. Hall, Ina L. Smith, Tanja Strive **Identification of novel hepaciviruses via pathogen profiling in North American lagomorphs.**	*Virus Evolution*	Published 02/07/2025	https://doi.org/10.1093/ve/veaf050
ISI Journal paper	Elena Smertina, Megan Pavy, Nias Y. G. Peng, Omid Fahri, Maria Jenckel, Philip Hands, Tanja Strive, Michael Frese, Ina L. Smith **Inhibition of interferon signalling improves rabbit calicivirus replication in biliary organoid cultures.**	*Journal of Virology*	Published 25/07/2025	https://doi.org/10.1128/jvi.00574-25

ISI Journal paper	HL Yeap, Á-D Popa-Baez, M Jenckel, S K Topfer, J Alves, P Andrade, M Carneiro, P Taggart, T Cox, S Campbell, K Patel, M Letnic, T Walsh, O R Edwards, R Hall, W T Tay, F Jiggins, R V Rane, I Smith, T Strive, K P Oh **Population genomics of invasive rabbits in Australia and prospects for genetic biocontrol at a continental scale**	*Journal TBA*		Draft manuscript attached as a separate file (Attachment B)

9.2 Other communications/activities (cumulative)

Date	Type	Description	Link
23/09/2020	Media release	A media release announced the commencement of the project through the CISS, MLA and Rabbit Free Australia comms	https://invasives.com.au/news-events/our-members-csiro-and-mla-developing-new-ways-to-control-wild-rabbit-populations/?utm_source=CISS+external+e-news+subscriber+list&utm_campaign=fbfd823df5-EMAIL_CAMPAIGN_2020_01_09_01_25_COPY_01&utm_medium=email&utm_term=0_dca65e59c7-fbfd823df5-93587421 http://www.rabbitfreeaustralia.com.au/research/search-to-begin-for-the-next-rabbit-bio-control/
24/03/2021	CISS Newsletter	CISS internal newsletter highlighting the successful development of rabbit intestinal organoids	https://invasives.com.au/news-events/could-organoids-help-optimise-rabbit-virus-management/
9/04/2021	Media	Media piece on rabbit organoids for biocontrol	https://www.farmonline.com.au/story/7199298/war-against-rabbits-will-never-end/
22/06/2021	The Immunology podcast	Rabbit organoids	https://immunologypodcast.com/ep-8-veterinary-virology-featuring-dr-robyn-hall
8/9/2021	Keynote presentation	Evolution and ecology of Australian rabbit caliciviruses	MicroSeq2021

11/11/2021	Invited talk	Evolution and ecology of Australian rabbit caliciviruses	Australian Society of Microbiology NSW/ACT branch meeting
21/3/2022	Invited talk	RHDV in the field Two scientists' perspectives	Rabbit Free Australia Rabbit R&D Update (with Pat Taggart)
1/3/2022	This Podcast Will Kill You	Rabbit Haemorrhagic Disease virus	https://thispodcastwillkillyou.com/2022/03/01/bonus-episode-rabbit-hemorrhagic-disease-virus/
5-8/12/2022	Poster	Hepatobiliary organoids derived from leporids support the replication of hepatotropic lagoviruses	11th Australasian Virology Society Meeting, Gold Coast AVS11 (2022) — Australasian Virology Society
5-8/12/2022	Poster	Hepatitis E virus in wild and domestic rabbits in Australia	11th Australasian Virology Society Meeting, Gold Coast AVS11 (2022) — Australasian Virology Society
19/9/2023	Media release	Bad hare day: New organoids boost pest rabbit control	https://www.csiro.au/en/news/all/articles/2023/september/rabbit-control-with-organoids
16-21/07/2023	Poster/ Short Talk	Meta-transcriptomic identification of novel Sylvilagus hepaciviruses via pathogen profiling in North American Lagomorphs	23rd International Congress of Genetics, "Genetics & Genomics: Linking Life & Society", Melbourne
06-07/09/2023	Talk	Meta-transcriptomic identification of novel Sylvilagus hepaciviruses via pathogen profiling in North American Lagomorphs	MicroSeq 2023, virtual Presentation won "Best Virology Talk"
17-21/09/2023	Poster	Using crosslinking proteomics to identify rabbit calicivirus receptor	Human Proteome Organisation (HUPO) world congress, Busan, Korea https://2023.hupo.org/
20/03/2024	Invited presentation	Rabbit Biocontrols – Current status and where next (T. Strive and B. Cooke)	Rabbit R&D Webinar organised by Rabbit Free Australia, ~ 180 virtual participants
May 2024	Poster	Towards novel genetic biocontrol for managing invasive rabbits in Australia	Gordon Research Conference on Genetic Biocontrol, Barcelona Spain.
29/05/2024	Invited presentation	Novel genetic biocontrol tools for the control of vertebrate pests in Australia – opportunities and challenges	Department of Agriculture, Fisheries and Forestry (DAFF) internal science seminar

30/07/2024	Talk	Pathogen detectives: Finding new tools and preparing for new threats	19th Australasian Vertebrate Pest Conference Session: Managing Rabbits https://www.youtube.com/watch?v=fCvGu4mVIAk&list=PLvvZSynxYkKfPjTvi5jAWjL9wbm5Lf-Y_&index=6&ab_channel=CentreforInvasiveSpeciesSolutions
31/07/2024	Talk	Gene drives in the wild: Using population genetics to inform development and evaluation of genetic biocontrols	19th Australasian Vertebrate Pest Conference Session: Biocontrol and biotechnology https://www.youtube.com/watch?v=JtAvjWMPGZE&list=PLvvZSynxYkKfrd1TVV77w5qXhbiwOlval&index=3&ab_channel=CentreforInvasiveSpeciesSolutions
31/07/2024	Talk	Enhancing rabbit biocontrol strategies through hepatobiliary organoid cultures and directed virus evolution	19th Australasian Vertebrate Pest Conference Session: Biocontrol and biotechnology https://www.youtube.com/watch?v=--p2iGV80Ys&list=PLvvZSynxYkKfrd1TVV77w5qXhbiwOlval&index=5&ab_channel=CentreforInvasiveSpeciesSolutions
31/10/2024	Online publication	Benchtop bunnies/Rabbit Organoids	Inclusion into the annual CSIRO Science highlights 2024 https://www.csiro.au/-/media/About/Files/24-00292_COMMS_REPORT_Highlights2023-24_WEB_241030.pdf
07/01/2025	TV Show	Eat the Invaders	Episode 1: Rabbit https://iview.abc.net.au/show/eat-the-invaders
19/3/2025	Invited presentation	A gene drive for rabbit control	Rabbit R&D Webinar hosted by Rabbit Free Australia
19/3/2025	Invited presentation	Dispersal of a gene drive for rabbit control	Rabbit R&D Webinar hosted by Rabbit Free Australia
16/06/2025	ABC News (various platforms	There was a time when Australia was overrun with rabbits in plague proportions and environmentalists say it's time to act again.	Multimedia/TV: https://www.abc.net.au/news/2025-06-16/professional-shooters-patrol-parks-in-canberra-in/105420674 Print: https://www.abc.net.au/news/2025-06-14/professional-shooters-patrol-parks-in-

			canberra/105401374?fbclid=IwY2xjawK8YShleHRuA2FlbQIxMQBicmlkETE1ZlJONUpxejdYNEM1MWpVAR7Vlg_NC3dr1XSekcnY1YGgV6Jva2-floV4DsdpeoaXPb9ulrgi8u6cMqZ0Kw_aem_z6eohCVKbR-zgj7-RzvU6g Social: https://www.instagram.com/p/DK8x3tYgxOY/
24/06/2025	The Land	What keeps biosecurity experts up at night?	Four biosecurity experts share their fight against invasives | The Land | NSW
14/08/2025	Invited presentation	What's in a name? Biocontrols versus biocides – what do we have and what do they do?	Presentation to the National Rabbit Managers Network, hosted by Rabbit Free Australia

10. Attachments (provided as separate files)

Attached to this report as separate compressed files are:

- **Attachment A:** Economic Assessment (Milestone 15): Impact evaluation of the implementation of the Rabbit Biocontrol Pipeline: RHD-Accelerator
- **Attachment B:** Draft manuscripts (submitted and in preparation)
- **Attachment C:** Previous annual reports 2021 to 2024.