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# DIFFERENT SEROLOGICAL PROFILES TO CO-OCCURRING PATHOGENIC AND NONPATHOGENIC CALICIVIRUSES IN WILD EUROPEAN RABBITS (*ORYCTOLAGUS CUNICULUS*) ACROSS AUSTRALIA

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ABSTRACT: Rabbit hemorrhagic disease virus (RHDV) was released in Australia as a biocontrol agent for wild European rabbits (*Oryctolagus cuniculus*) in 1995–96; however, its effects were variable across Australia with the greatest population reductions seen in lower annual rainfall areas (<400 mm). There is speculation that the reduced effectiveness observed at higher annual rainfall sites is at least partially due to the presence of a nonpathogenic calicivirus (RCV-A1). The RCV-A1 is related to RHDV and confers partial and transient protection against lethal RHDV infection in laboratory tests. What is not well understood is where, how, and to what degree RCV-A1 impedes the effect of RHDV-mediated rabbit control under field conditions. We investigated seven wild rabbit populations across six states and territories representing different seasonal rainfall zones across Australia, four times during 2011–12, to investigate if the presence and prevalence of RCV-A1 coincided with a change in RHDV immunity status within these populations. Besides serology, tissue samples from both trapped and shot rabbits were collected for virus detection by reverse transcription PCR. Overall, 52% ($n$=258) of the total samples ($n$=496) tested positive for RHDV antibodies and 42% ($n$=208) positive for RCV-A1 antibodies; 30% ($n$=150) of the sera contained antibodies to both viruses. The proportion of rabbits with RHDV antibodies increased significantly at sites where RCV-A1 antibodies were present ($\chi^2_1$, $\alpha$=0.1, $P$<0.001). Evidence that preinfection of RCV-A1 may lead to a higher proportion of sampled rabbits with antibodies to both viruses was found at only one site.

*Key words:* Biocontrol, disease, epidemiology, introduced, resistance, RHDV, wildlife.

## INTRODUCTION

Rabbit hemorrhagic disease virus (RHDV) is a lagovirus (Family *Caliciviridae*) that causes rabbit hemorrhagic disease (RHD), a fatal infectious hepatitis with very high case fatality rates in European rabbits (*Oryctolagus cuniculus*) reviewed in Abrantes et al. (2012). Rabbit hemorrhagic disease virus was first described in domestic rabbits in China (Liu et al. 1984). A strain of RHDV (Czech 351) was imported into Australia in 1991 and tested in quarantine to assess its usefulness as a biological control agent for overabundant rabbits (Cooke and Fenner 2002). The virus escaped from quarantine in 1995 and was later released in a nationwide coordinated program in 1996 (McPhee et al. 2009; Mutze et al. 2010). The effects of RHDV on reducing rabbit numbers varied across Australia. Rabbit population reductions of up to 95% were seen in arid regions (Bowen and Read 1998; Mutze et al. 1998). Rabbit hemorrhagic disease virus had less impact in higher rainfall areas with rabbit reductions between 0% and 40% (Saunders et al. 1999). Rabbits in the higher rainfall areas with increased survival rates were shown to have antibodies cross-reacting to RHDV (Nagesha et al. 2000; McPhee et al. 2009). Based on these serological profiles, a nonpathogenic rabbit calicivirus that provided immunological cross-protection against lethal RHD infection was speculated to circulate in wild rabbits (Cooke et al. 2002; Robinson et al. 2002). Rabbit Calicivirus Australia 1 (RCV-A1) was first described in wild rabbits in 2009

TABLE 1. Locations and rainfall characteristics of seven sites in Australia sampled for European rabbit (*Oryctolagus cuniculus*) density and epidemiology between April 2011 and February 2012 to investigate if the presence and prevalence of the Australian benign calicivirus RCV-A1 coincided with a change in rabbit hemorrhagic disease virus immunity status within these populations. All sites were sampled in the months of April, July, October, and January except for Muncoonie and Stirling, which were only sampled in the months of July, October, and January because of lack of access in April.

| Site | State[a] | Coordinates | Mean annual rainfall (mm) | Mean winter rainfall (mm) | Mean annual temperature (C) |
|---|---|---|---|---|---|
| Muncoonie | QLD | 25°13′30″S, 138°37′59″E | 128.8±79.8 | 7.2±39 | 31.5±2.4 |
| Euchareena | NSW | 32°58′59″S, 149°13′30″E | 562.0±35.5 | 50.4±43.1 | 22.3±3.6 |
| Oaky Creek | NSW | 33°24′40″S, 149°22′1″E | 758.7±236.4 | 70.6±49.8 | 19.9±3.1 |
| Hattah | VIC | 34°36′29″S, 142°18′11″E | 308.1±333.0 | 27.6±48.3 | 23.7±1.7 |
| Coorong | SA | 35°12′0″S, 139°39′36″E | 519.0±198.3 | 73.8±22.3 | 20.8±1.2 |
| Erldunda | NT | 25°36′22″S, 133°10′41″E | 249.9±433.0 | 12.5±90.4 | 27.5±1.0 |
| Stirling | WA | 34°31′59″S, 118°21′58″E | 728.1±261.3 | 130.1±104.9 | 24.8±1.3 |

[a] QLD = Queensland; NSW = New South Wales; VIC = Victoria; SA = South Australia; NT = Northern Territory; WA = Western Australia.

(Strive et al. 2009). The RCV-A1 causes an asymptomatic infection in rabbits but can confer partial and transient protection against lethal RHDV infection (Strive et al. 2010, 2013).

The prevalence of RCV-A1 was recently mapped across Australia. Its distribution was confirmed to be predominantly associated with cool temperatures and high rainfall areas (Liu et al. 2014), where the effectiveness of RHDV was limited (Saunders et al. 1999; Richardson et al. 2007). However, the interaction between RHDV and RCV-A1 in wild rabbit populations and the impact of RCV-A1 presence on the effectiveness of RHDV as a biological control agent are not yet well understood. We hypothesized that in areas where previous RCV-A1 infection is partially responsible for surviving RHD, seroprevalence to both viruses would be increased. To this end, we investigated the demographic and serological profiles of RHDV and RCV-A1 in rabbit populations across Australia.

## MATERIALS AND METHODS

### Sampling of rabbits

We monitored seven sites across six states and territories representing three rainfall zones across Australia once in the middle of each season (April in autumn, July in winter, October in spring, and January in summer) from April 2011 to January 2012 (Table 1 and Fig. 1). Rabbits were either shot or trapped ($n$=496). Shooting was carried out by licensed shooters with a 0.22 caliber rifle according to Standard Operating Procedures for Ground Shooting of Rabbits (http://www.pestsmart.org.au/ground-shooting-of-rabbits/), targeting the head or chest. Blood (for sera), liver (for active RDHV infection), duodenum (for active RCV-A1 infection), and eyeball (for animal age) were collected from up to 20 shot animals during each sampling period at each site. Liver and duodenal tissues were stored frozen at –20 C, and eyeballs were collected in 10% neutral buffered formalin.

We undertook cage trapping at multiple rabbit warrens at the Euchareena ($n$=10) and Oaky Creek ($n$=12) sites in New South Wales (NSW). One hundred wire cage traps (200×200×80 mm) were placed at burrow entrances of warrens at each site. We did not undertake cage trapping at other sites due to the limit of available resources. We covered half of each trap to provide weather protection for trapped animals and as a visual barrier against predators. We baited traps with freshly diced carrot each day and used a minimum of a 1 wk free-feed period to encourage rabbits onto the bait. Trapping occurred over four nights during each sampling session. Rabbits caught on days 1–3 were ear-tagged; rabbits heavier than 400 g had blood taken from the ear vein, and all rabbits were released. Rabbits captured on day 4 were euthanized via stunning using a captive bolt (Item no. 9023200, Friedr. Dick GmbH & Co., Esslingen, Germany) followed by cervical dislocation. Liver, duodenum, and eyeballs were sampled, and blood was collected from any new capture. This research

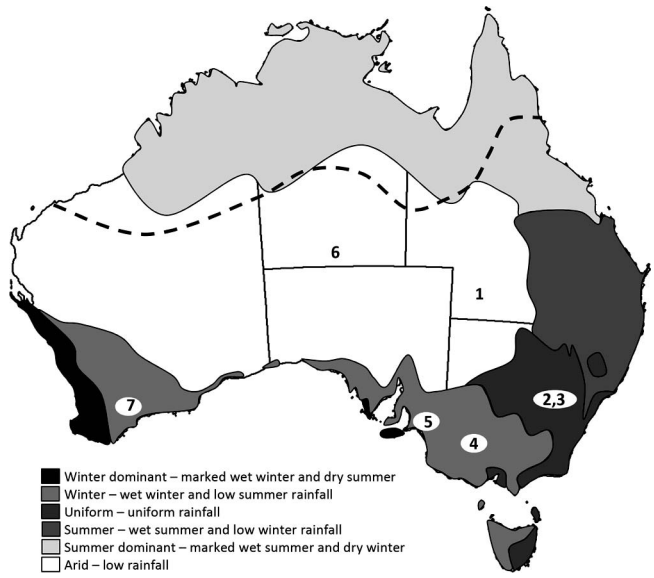


FIGURE 1. The major seasonal rainfall zones of Australia and the location of the seven sites where European rabbits (*Oryctolagus cuniculus*) were sampled for the Australian benign calicivirus RCV-A1 and rabbit hemorrhagic disease virus antibody presence: 1) Muncoonie Lakes, Queensland; 2) Euchareena, New South Wales; 3) Oaky Creek, New South Wales; 4) Hattah-Kulkyne National Park, Victoria; 5) Coorong, South Australia; 6) Erldunda Station, Northern Territory; 7) South Stirling Ranges, Western Australia with the dashed line indicating the northern limits of European rabbit distribution in Australia (Commonwealth of Australia 2005).

was undertaken under the Animal Research Authority ORA 11/14/001.

### Sample analysis

We determined the serological status for RHDV by using isotype enzyme-linked immunosorbent assays (ELISAs) that detect immunoglobulin G, immunoglobulin A, and immunoglobulin M (IgM) antibodies and competition ELISAs to measure the concentration of antigen (Capucci et al. 1991, 1997) and for RCV-A1 by using competition ELISA (Liu et al. 2012). In populations where RCV-A1 antibodies were present, we attempted to detect active RCV-A1 infections in duodenum samples. For this purpose, approximately 20 mg from each of five duodenum samples were pooled, and the RNA was extracted using TRIzol® Reagent (Sigma Aldrich, Sydney, Australia), according to the manufacturer's instructions. Quantitative reverse transcription PCR (qRT-PCR) was carried out as described previously (Hoehn et al. 2012).

Eyeballs were used to classify rabbits by age. Whole eyeballs were stored in 10% neutral buffered formalin for a minimum of 28 d and processed as described previously to calculate age-to-the-day for the rabbits (Augusteyn 2007). Where eye lens weight was not available (due to noncollection), body weight was used as an indicator of age. While it is common to divide rabbit age classes into three categories: kitten (<900 g), subadult (900–1200 g), and adult (>1200 g), in regard to disease susceptibility any protective maternal antibodies have usually completely dissipated by 12 wk of age (Robinson et al. 2002). Therefore, we divided rabbits into two age classes: kittens (up to 12 wk old or <900 g in body mass) and adults (>12 wk old or >900 g in body mass).

### Statistical analysis

We modeled our findings as multinomial data in an additive combination of effects as baseline+ Status+State:Status+Site:Status+Season:Status+ State:Season:Status, where Status is the antibody status of the animal, Site is the sampling location, State is the state the site is in (e.g., NSW or Queensland-QLD), and Season is the time of year (autumn, winter, spring, summer). Log-linear modeling was used with the observations treated as (quasi-) Poisson realizations but with the constraint that the totals for each Site×Season were fixed. The model was fitted in R (R Core Development Team 2010) using the *glm* function. The quasi-Poisson model was used to allow for possible overdispersion.

To test if a rabbit was more or less likely to have RHDV immunity at a site with RCV-A1 as

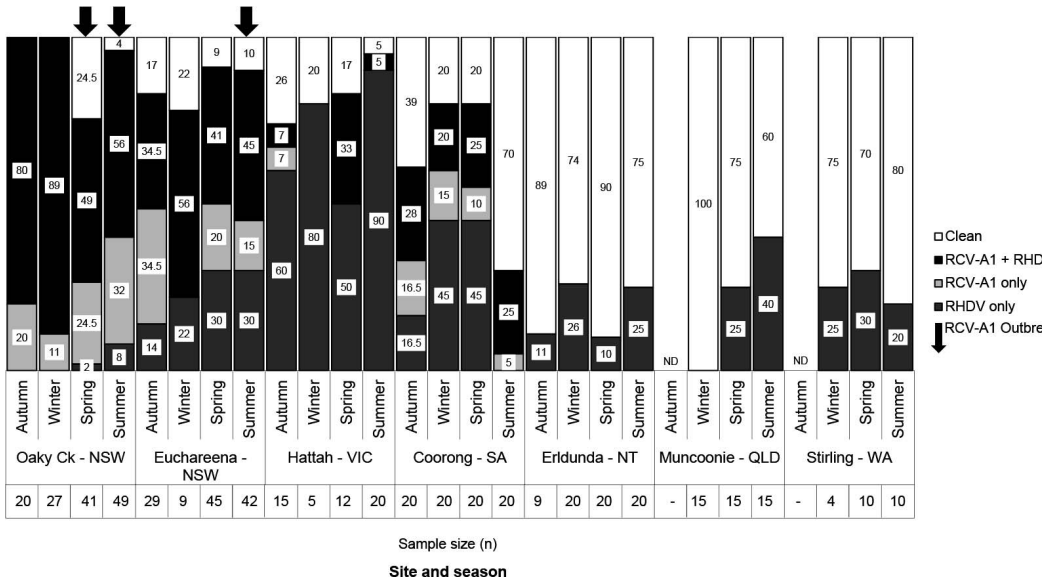


FIGURE 2. The percentage of European rabbits (*Oryctolagus cuniculus*) in Australia with each antibody type: rabbit hemorrhagic disease virus (RHDV) only (adult and maternal), Australian benign calicivirus RCV-A1 only or both RHDV and RCV-A1, and Clean rabbits in each population sampled over summer, autumn, winter, and spring at seven sites between April 2011 and February 2012, including sample sizes. Down arrows indicate positive quantitative reverse transcription PCR results suggesting an active RCV-A1 infection. NSW=New South Wales; VIC=Victoria; SA=South Australia; NT=Northern Territory; ND=no data; QLD=Queensland; WA=Western Australia.

compared to a site without RCV-A1, and if this likelihood would change across seasons within Sites, a logistic mixed model analysis was performed. Letting $p$ denote the probability a trapped rabbit has immunity to RHDV, the full model for logit($p$), that is, $\log(p/(1-p))$, is logit($p$)=baseline+RCVatSite+Season+RCVatSite:Season+***Site***+***Site:Season***. In this model, RCVatSite was an indicator variable, indicating the presence or absence of RCV at the site, while terms in bold/italic were fitted as random effects. The model was fitted using the *glmer* function in package *lme4* (Bates et al. 2011) in R.

## RESULTS

### Seroprevalence of RHDV and RCV-A1

Over the four sampling sessions, 504 rabbits were shot ($n$=415) or trapped ($n$=89) resulting in the collection of 496 blood samples, 504 liver samples, 502 duodenum samples, and 459 eye lenses. Overall, 64% (319/496) of rabbits had a positive antibody response (Fig. 2). Eighty-eight percent of the rabbits sampled for blood were adults, and of these, 64% ($n$=282) had a positive serological response. A total of 13% (of the rabbits analyzed) were kittens, of which 64% ($n$=37) had a positive antibody response.

### Detection of recent or active virus infection

We detected IgM antibody against RHDV (indicating a recent exposure of the population for RHDV) in samples from 11 animals: two adults at Oaky Creek in April 2011 and October 2011, one adult at Coorong in October 2011, and eight rabbits (one adult and seven kittens) at Euchareena in October 2011. All the RHDV-IgM–positive adults were positive for RCV-A1 antibodies. In contrast, six out of the seven RHDV-IgM–positive kittens at Euchareena were negative for RCV-A1 antibodies, indicating an RHDV infection prior to RCV-A1 infection. The presence of RCV-A1 viral RNA in duodenum samples was detected by qRT-PCR in at least four samples from Oaky Creek and Euchareena in October 2011 and January 2012, indicating RCV-A1 virus activity during this

TABLE 2. Analysis of deviance of the European rabbit (*Oryctolagus cuniculus*) Australian benign calicivirus RCV-A1 status across seven sites within five jurisdictions in Australia between April 2011 and February 2012 to investigate if the presence and prevalence of RCV-A1 coincided with a change in rabbit hemorrhagic disease virus immunity status within these populations, and whether location and season were a contributing factor. Terms were added sequentially (first to last).[a]

| Model | Df | Deviance | Resid df | Resid dev | *F* | Pr(>*F*) |
|---|---|---|---|---|---|---|
| Null | | | 103 | 529.48 | | |
| Status | 3 | 48.91 | 100 | 480.57 | 6.488 | 0.007 |
| Status:State | 20 | 368.43 | 80 | 112.14 | 7.331 | 0.001 |
| Status:Site | 4 | 15.13 | 76 | 97.01 | 1.505 | 0.262 |
| Status:Season | 12 | 11.69 | 64 | 85.32 | 0.388 | 0.943 |
| Status:State:Season | 52 | 52.70 | 12 | 32.61 | 0.403 | 0.988 |

[a] df = degrees of freedom; resid df = residual degrees of freedom; resid dev = residual deviance.

period. Five duodenum samples were pooled in this assay, and all pools that tested positive for viral RNA consisted predominantly of kitten samples.

To test if there was serological evidence that the presence of RCV-A1 increased the likelihood that a greater proportion of the population would have antibodies to RHDV, we compared the numbers of rabbits in four serological categories: Clean (no antibodies to RHDV or RCV-A1), RHDV (antibodies to RHDV only), RCV-A1 (antibodies to the RCV-A1 only), and Both (antibodies to both RHDV and RCV-A1). If infection with RCV-A1 was protecting rabbits from acute RHD, there would be a higher-than-expected frequency of the rabbits classed as Both. That is, the ratio of Both/RCV-A1 should be higher than the ratio of RHDV/Clean. Fitting the model (Table 2), the interaction effect Status:Site was significant ($P$=0.012) after sequentially dropping Status:State:Season and then Status:Season, implying that the two NSW sites, Euchareena and Oaky, differed in their probabilities that animals would fall within each of the four abovementioned categories. The probabilities that an animal would fall within each of four categories differed across sites, but did not differ significantly across seasons within sites (Table 2).

Fitting a model that was independent of Season, sites without commonality in the least significant difference rank differed significantly (Table 3). The sites, Erldunda, Muncoonie, and Stirling had significantly higher proportion of rabbits classified as Clean than other sites, and the Hattah site had a significantly

TABLE 3. Probabilities and their ±SE of a European rabbit (*Oryctolagus cuniculus*) falling into a particular epidemiological category across seven sites across Australia. Sites without a capital letter in common differ significantly (least significant difference, $\alpha$=0.05).[a]

| Site | Clean (SE) | RHDV (SE) | RCV-A1 (SE) | Both (SE) |
|---|---|---|---|---|
| Coorong | 0.36 (0.07) B | 0.28 (0.07) B | 0.17 (0.05) AB | 0.19 (0.06) A |
| Erldunda | 0.81 (0.12) C | 0.19 (0.06) B | 0.00 AB | 0.00 AB |
| Euchareena | 0.12 (0.04) A | 0.17 (0.05) B | 0.26 (0.05) B | 0.45 (0.07) B |
| Hattah | 0.15 (0.06) A | 0.71 (0.13) C | 0.02 (0.05) A | 0.12 (0.06) A |
| Muncoonie | 0.76 (0.13) C | 0.24 (0.07) B | 0.00 AB | 0.00 AB |
| Oaky | 0.09 (0.03) A | 0.04 (0.02) B | 0.24 (0.05) B | 0.64 (0.08) B |
| Stirling | 0.75 (0.19) A | 0.25 (0.12) A | 0.00 AB | 0.00 AB |

[a] clean = no antibodies to rabbit hemorrhagic disease virus (RHDV) or the Australian benign calicivirus RCV-A1; RHDV = antibodies to RHDV; RCV-A1 = antibodies to the Australian benign calicivirus; both = antibodies to both RHDV and RCV-A1.

TABLE 4. Estimates of the probabilities (*P*) for a European rabbit (*Oryctolagus cuniculus*), at four of the seven sites sampled in Australia where the Australian benign calicivirus RCV-A1 is present, having antibodies to rabbit hemorrhagic disease virus (RHDV) given (1) antibodies to RCV-A1 and (2) no antibodies to RCV-A1.

| Viruses | Probability of antibodies present | | | |
|---|---|---|---|---|
| | Coorong | Euchareena | Hattah | Oaky |
| RHDV and RCV-A1 | 0.54 | 0.63 | 0.86 | 0.73 |
| RHDV but no RCV-A1 | 0.44 | 0.60 | 0.82 | 0.82 |

higher proportion of rabbits classified as RHDV (Table 3 and Fig. 2).

Taking this analysis to the site level, and looking only at sites where RCV-A1 antibodies were present, we estimated the probabilities for a rabbit having antibodies to RHDV given 1) antibodies to RCV-A1 and 2) no antibodies to RCV-A1 (Table 4). For each site the probability of RHDV antibodies given a rabbit had antibodies to RCV-A1 was larger than the probability of RHDV antibodies given a rabbit had no RCV-A1 antibodies. However, this was significant only at Oaky ($\chi^2_1$, $\alpha$=0.05, $P$=0.002).

All seven sites we sampled had animals with antibodies to RHDV. Four of these sites also had animals with RCV-A1 antibodies present. The proportion of RHDV antibody-positive rabbits increased significantly at sites (except Hattah) where RCV-A1 was present ($\chi^2_1$, $\alpha$=0.1, $P<0.001$) (Fig. 3). The probability of sampling a rabbit with antibodies to RHDV was greater at sites where RCV-A1 was present (0.66±0.05 SE) than where RCV-A1 was absent (0.20±0.05 SE).

## DISCUSSION

We found a strong correlation between the presence of RCV-A1 antibodies and RHDV antibodies. At all sites where RCV-A1 was present we were significantly more likely to find animals with RHDV antibodies. This was true at both a national level and at a site level, although only at the Oaky site was the probability of finding a rabbit with RHDV antibodies significantly more likely if the rabbit had RCV-A1 antibodies than if it didn't. The Oaky site is the only site where RCV-A1 antibodies were detected in 100% of the samples during any of the sampling sessions (autumn and winter; Fig. 2). The Oaky site is a cool-wet site and the wettest site with the

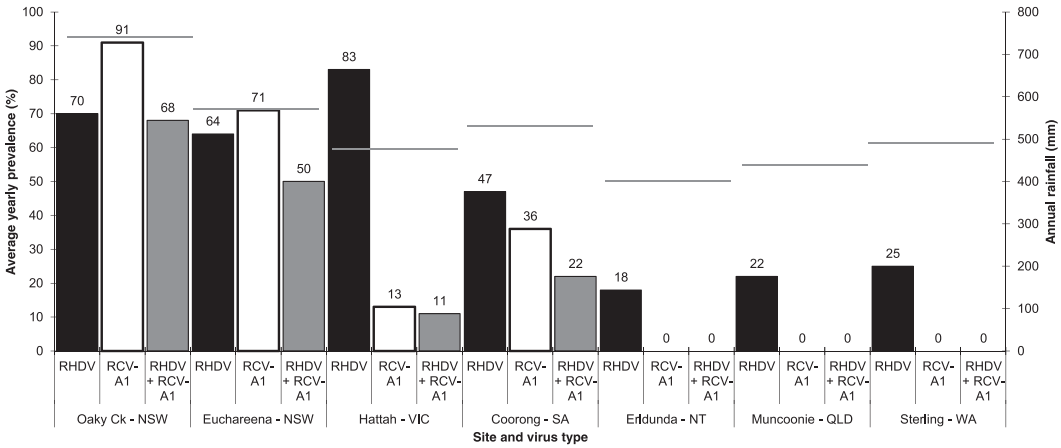


FIGURE 3. The average yearly prevalence of rabbit hemorrhagic disease virus (RHDV), Australian benign calicivirus RCV-A1, and both RHDV and RCV-A1 in European rabbits (*Oryctolagus cuniculus*) in Australia across seven sites between April 2011 and February 2012 and the average annual rainfall for each site. NSW=New South Wales; VIC=Victoria; SA=South Australia; NT=Northern Territory; QLD=Queensland; WA=Western Australia.

highest winter rainfall in this study (mean annual rainfall=758.7±236.4 mm; mean annual winter rainfall=70.6±49.8 mm), and mean annual maximum temperature of 19.9±3.1 C. Liu et al. (2014) reported strong statistical support for RCV-A1 prevalence as a function of temperature and rainfall and that sites with rabbits with a high prevalence (>50%) of RCV-A1 antibodies had a mean annual rainfall of 608±179 mm. Oaky was also the site where rabbits with active RCV-A1 infections were detected via qRT-PCR in spring and summer. It is possible that the Oaky site differs from other sites due to very high rainfall, particularly in winter, and that, at these very high winter rainfall/low mean temperature sites, the impact of the two viruses is not independent.

It is not clear if the increased RHDV seroprevalence at our sites is a direct result of high RCV-A1 prevalence. The prevalence of RCV-A1 is highest in areas of above average rainfall, conditions that also support high rabbit densities and year-round breeding of at least some individuals. It is feasible that the constant presence of young rabbits innately resistant to lethal RHDV infection (Morisse et al. 1991; Robinson et al. 2002) alone may be sufficient for reduced mortality during an RHDV outbreak, regardless when it occurs. However, a constant supply of young rabbits would also favor constant exposure of these young animals to RCV-A1, and any transient protective effect of a previous RCV-A1 infection may help extend the period of increased survival probability in rabbit kittens. This would explain the observations made at the Oaky Creek site, where 73% to 100% of rabbits tested positive for RCV-A1 antibodies throughout the year, and RCV-A1 was detected at this site every time kittens were sampled. It is therefore possible that a high prevalence of RCV-A1 does contribute to the increased survival rates and the high occurrence of RHDV-immune animals, but it is not clear to what extent and further investigation is required.

Where RCV-A1 prevalence was low, RHDV seroprevalence was also low, except at the Hattah site. The seroprevalence of RCV-A1 at Hattah is as low as it is in the arid sites of Erldunda and Muncoonie, yet the proportion of animals at Hattah with antibodies to RHDV is the highest out of all the sites sampled. At the Hattah site, the presence of RCV-A1 is not the likely cause of the high prevalence of RHDV antibodies. Low RCV-A1 and high RHDV prevalence would suggest a mechanism other than RCV-A1 exposure to maintain such high frequency of surviving animals. Hattah is a comparatively arid site where year-round breeding of rabbits is unlikely; the constant exposure of innately resistant young at this site appears an unlikely cause for the high seroprevalence. Nyström et al. (2011) and Elsworth et al. (2012) have identified the Hattah population of rabbits as a population with increased genetic resistance to RHDV infection. Nyström et al. identified histo blood group antigens (HBGAs) as coreceptors for RHDV infection. Rabbits without the correct HBGA ligands were more resistant to infection with RHDV at low doses, and survivors of RHDV outbreaks in wild rabbit populations showed increased frequency of weak-binding phenotypes, suggesting that HBGAs could contribute to genetic resistance at the population level. Nyström et al. identified the Hattah population as one with an increased frequency of weak-binding phenotypes and therefore possible genetic resistance. The high RHDV antibody prevalence (73–95%) and low RCV-A1 prevalence (0–33%) suggested that protection by RCV-A1 was not likely a major factor in the high rate of RHDV seroconversion. Notably, the genetic resistance mechanism suggested by Nyström et al. would likely result in avoidance of productive infection with RHDV and, consequently, a lower seroprevalence in the population. However, high RHDV seroprevalence suggests increased survival rates at this site, indicating the possibility of additional mechanisms facilitating resistance to disease, not just to infection. It is possible that a serological profile of rabbits similar to that seen at Hattah (low RCV-A1 prevalence, very high RHDV prevalence) could be used as a quick and relatively cheap indicator to prescreen and help identify other possible genetic resistant

populations within Australia (Fig. 3). Populations with this serological profile could then be further investigated for genetic resistance.

The analysis of the serological status of rabbits from seven sites across Australia showed that 64% of sampled rabbits had antibodies for RHDV, RCV-A1, or both. This suggests that, averaged over Australia, more than half of all adult rabbits are potentially immune to a lethal infection of the current strain of RHDV used in Australia to control rabbits, although levels of immunity would vary greatly depending on the geographic location and recruitment. While sampling by shooting is skewed toward adults, our sampling by trapping captured mainly kittens. The sampled kittens also showed high levels of antibodies to RHDV, RCV-A1, or both, indicating that, at least in the tablelands of NSW, approximately one third of the next generation of rabbits could already have acquired immunity to lethal RHDV infection at a young age. Mutze et al. (2014) reported that kittens <600 g were being killed by RHDV at Turretfield in South Australia (where RCV-A1 antibodies are present at low prevalence; Liu et al. 2014) and that increased juvenile infection contributes to the recovery of rabbit populations. Mutze et al. (2014) suggested that the observed high rate of kitten survival may drive rabbit population recovery at this site, an assertion supported by Calvete (2006), whose population model found that a lower age of infection drove rabbit population recovery.

Liu et al. (2014) reported RCV-A1 was present in kittens as young as 3 wk old, and it is feasible that the presence of RCV-A1 in the kittens of these populations influence RHD epidemiology and contributes to the long-term recovery of rabbit populations. However, further studies investigating the dynamics of both infections will be required to confirm this hypothesis. Longitudinal monitoring of outbreaks of both viruses over time will be needed to ascertain if the serological profiles to RHDV change when a RCV-A1 outbreak precedes an RHDV outbreak in a population.

Recent work showed that RCV-A1 serum antibodies alone are not protective against RHDV (Strive et al. 2013). Instead, cellular immune mechanisms were suggested to be responsible for the transient cross-protection. In our study, qRT-PCR analysis detected RCV-A1 on only three occasions at two sites during the sampling period. No outbreaks were recorded at any of the other sites where RCV-A1 antibodies were present. A positive PCR result detects an active virus infection and is therefore indicative for an RCV-A1 outbreak at the site. All three pools that tested positive in the RCV-A1 PCR consisted predominantly of kitten samples. At Oaky Creek, every time kittens were sampled they tested positive for an active RCV-A1 infection. The RCV-A1 was first isolated from rabbit kittens (Strive et al. 2009), and work by Richardson et al. (2007) showed that the majority of rabbits acquire antibodies against RCV-A1 by the time they reached 1,000 g, indicating an RCV-A1 infection within the first 3 mo of their life. Therefore, it is likely that the detection rate of RCV-A1 infection was greatly reduced in our study by the strong bias toward adult animals in the sample. Studies investigating the seasonality of RCV-A1 will need to specifically target rabbit kittens at these sites.

In conclusion, the serological profiles for both RHDV and RCV-A1 varied greatly between sites, suggesting that dynamics and interactions of RHDV and RCV-A1 may have varied greatly depending on a variety of environmental factors, and highlighting the need for widespread sampling to cover a broad range of climatic regions in Australia. Rabbits were likely to have a higher proportion of antibodies to RHDV in populations where RCV-A1 was present; however, we detected only a statistically significant finding that previous RCV-A1 exposure led to a higher proportion of RHDV-positive rabbits at one site. While this finding supported historical observations that RCV-A1 at high prevalence can impede the effectiveness of RHDV, more longitudinal cohort studies of populations where these viruses coexist are required.

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