Retrospective serological analysis reveals presence of the emerging lagovirus RHDV2 in Australian wild rabbits at least six months prior to its first detection.

Running title: Serological evidence for earlier RHDV2 arrival in Australia

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Summary:

The lagovirus Rabbit Haemorrhagic Disease Virus (RHDV) has been circulating in Australia since the mid-1990s when it was deliberately released to control overabundant rabbit populations. In recent years, the viral diversity of different RHDVs in Australia has increased, and currently four different types of RHDV are known to be circulating. To allow for ongoing epidemiological studies and impact assessments of these viruses on Australian wild rabbit populations, it is essential that serological tools are updated. To this end, Reference sera were produced against all four virulent RHDVs

(including RHDV2) known to be present in Australia and tested in a series of available immunological assays originally developed for the prototype RHDV, to assess patterns of cross reactivity and the usefulness of these assays to detect lagovirus antibodies, either in a generic or specific manner. Enzyme Linked Immuno Sorbent Assays (ELISAs) developed to detect antibody isotypes IgM, IgA and IgG were sufficiently cross reactive to detect antibodies raised against all four virulent lagoviruses. For the more specific detection of antibodies to the antigenically more different RHDV2, a competition ELISA was adapted using RHDV2 specific monoclonal antibodies in combination with Australian viral antigen. Archival serum banks from a long term rabbit monitoring site where rabbits were sampled quarterly over a period of six years were re-screened using this assay, and revealed serological evidence for the arrival of RHDV2 in this population at least six months prior to its initial detection in Australia in a deceased rabbit in May 2015. The serological methods and reference reagents described here will provide valuable tools to study presence, prevalence and impact of RHDV2 on Australian rabbit populations; however the discrimination of different antigenic variants of RHDVs as well as mixed infections at the serological level remains challenging.

Keywords:

Differential diagnostic, serology, RHDV, RHDVa, RHDV2, epidemiology, ELISA, biological control

Introduction:

Rabbit Haemorrhagic Disease Virus (RHDV, sometimes also referred to as RHDV1 or GI.1c, according to a new proposed nomenclature (Le Pendu et al., 2017)), belongs to the genus lagovirus within the family *caliciviridae*. RHDV was released in Australia as a biological control agent for introduced wild rabbits, a devastating agricultural and environmental pest species in this country (Cooke and Fenner, 2002). While initially very effective in reducing rabbit populations across large parts of the continent

(Saunders et al., 2002, Mutze et al., 1998), RHDV was not effective in the more temperate areas of South Eastern Australia. This lack of effectiveness was likely due to the presence of endemic, non-pathogenic caliciviruses (Rabbit calicivirus Australia 1, also termed RCV-A1, or GI.4) that can provide transient and partial immunological cross protection to RHDV (Strive et al., 2013, Liu et al., 2014), thereby reducing both case fatality and infection rates (Cooke et al., 2018). In addition to the impeding effects of RCV-A1, rabbit populations have been recovering in recent years (Mutze et al., 2014), and developing genetic resistance to RHDV has been reported in some Australian rabbit populations (Elsworth et al., 2012, Nystroem et al., 2011). In an attempt to 'boost' rabbit biocontrol in Australia and to maintain the substantial economic and environmental gains made by the long term suppression of rabbit populations by RHDV (Pedler et al., 2016, Cooke, 2013), an additional strain of RHDV was released nationwide in Australia in March 2017 (Hall et al., 2018, Strive and Cox, 2019). This strain termed RHDVa-K5 is a naturally occurring antigenic variant of RHDV from Korea (Oem et al., 2009) which was shown experimentally to be more effective in infecting rabbits from a genetically resistant rabbit population (Elsworth et al., 2012) and in overcoming partial protection conveyed by the benign RCV-A1 (Cox et al., 2013). These antigenic variants of RHDV, referred to as RHDVa (GI.1a, or RHDV1a), were first reported in the late 1990s (Capucci et al., 1998), and although they exhibit antigenic differences they are considered to be of the same serotype (Lavazza and Capucci, 2016).

Prior to the release of RHDVa-K5, the incursions of two additional RHDV variants were reported in Australia. The first incursion was another variant RHDVa (GI.1a) strain in the greater Sydney area that most closely resembled a Chinese isolate (Wang et al., 2012) that appeared in early 2014. This virus, termed RHDVa-Aus, caused a number of recorded outbreaks mostly in domestic rabbit farms, however, its distribution appeared geographically limited to the east and north east of New South Wales (Mahar et al., 2018b). In May 2015, the incursion of a second exotic virus, the recently emerged RHDV2 (GI.2) was reported in Australia (Hall et al., 2015). RHDV2 is a new lagovirus that was first reported in Europe in 2010 (Le Gall-Recule et al., 2011, Dalton et al., 2012). It is not only

genetically distinct from RHDV and RHDVa but, unlike RHDV and RHDVa, is also able to cause highly fatal disease in very young rabbits (Neimanis et al., 2018a, Dalton et al., 2014) and capable of fatally infecting several species of hares, making it the only known lagovirus that does not exhibit strict species specificity (Camarda et al., 2014, Hall et al., 2017, Le Gall-Recule et al., 2017, Neimanis et al., 2018b, Puggioni et al., 2013, Velarde et al., 2017). Since its arrival in Australia, RHDV2 has become the dominant strain circulating in the field, seemingly replacing older RHDV1 strains and accounting for the majority of reported cases in wild and domestic rabbits (Mahar et al., 2018a).

With the increasing diversity of co-circulating RHDVs both in Australia and Europe it was essential to update the diagnostic tools available, to allow for specific identification of strains and to measure the impact they were having on wild rabbit (and hare) populations. Due to the genetic variability, the development of specific molecular diagnostics was comparably straightforward, and accordingly several tailored approaches have been described in both Europe and Australia (Carvalho et al., 2017, Duarte et al., 2015, Hall et al., 2018, Le Gall-Recule et al., 2017). In contrast, the development of specific serological tools is more challenging. Although strain specific epitopes have been described for lagoviruses and specific monoclonal antibodies have been developed (Capucci et al., 1996a, Capucci et al., 1998, Capucci et al., 1995, Dalton et al., 2018, Kong et al., 2016, Liu et al., 2012b), discriminatory serology remains difficult, in particular for closely related RHDVs, due to the large number of shared epitopes (Lavazza and Capucci, 2016). The infection history of populations can often only be inferred based on reactivity patterns (Barcena et al., 2015, Velarde et al., 2016).

In Australia, the development of specific serological tools to discriminate between different lagoviruses was of particular importance. Here, RHDV2 was actively circulating at the time the new strain RHDVa-K5 was released nationwide (Strive and Cox, 2019) and specific serological tools were needed to investigate the respective impacts and potential interactions the two strains had on wild Australian rabbits at a population level. Furthermore, in the years leading up to the national release of RHDVa-K5, extensive serological monitoring of several Australian rabbit populations was carried

98 out. Screening these sample banks with a serological assay specific to RHDV2 might allow a more
99 accurate determination of the exact time point this virus entered into Australia and started to
100 circulate in wild rabbit populations before it was detected in May 2015 (Hall et al., 2015).

101 This study describes the production of reference sera raised against all virulent RHDV strains known
102 to be circulating in Australia for the purpose of assessing the cross-reactivity patterns in a panel of
103 existing serological assays used to infer disease dynamics of RHDV in rabbit populations. It further
104 describes the adaptation of a European RHDV2 cELISA to the Australian strain of RHDV2, and the
105 modification of IgM and IgA isotype ELISAs for the improved detection of RHDV2 antibodies. The
106 RHDV2 cELISA was then applied to retrospectively analyse a long term field monitoring site to
107 estimate the time of arrival on RHDV2 at this site.

108

109 **Material and methods:**

110 *Ethics approval:*

111 All work involving animals was approved by the CSIRO Ecosystem Sciences Animal Ethics Committee
112 (ESAEC #10-13, #11-01, #13-01, #13-10, #DOMRAB) and the Orange Animal Ethics Committee
113 (ORA11/14/001, ORA14/17/001) and carried out in accordance with the Australian code for the care
114 and use of animals for scientific purposes.

115 *Production of antigen, virus inoculum and experimental vaccine*

116 Two five week old New Zealand white rabbits were infected with 0.5 ml of a 2% clarified liver
117 homogenate of the first RHDV2 field isolate found in Australia (BlMt-1, Gen Bank# KT280060) (Hall et
118 al., 2015). Rabbits were monitored twice daily for signs of terminal rabbit haemorrhagic disease
119 (RHD), which was defined as 10% weight loss within a 24 hour period, no resistance to handling or
120 lateral recumbency, or hypothermia following a fever episode, often in combination with lethargy.
121 Both rabbits experienced a peracute form of RHD, one was found displaying signs of terminal RHD at

66 hours post infection (h.p.i.) and was euthanized, the second was found dead 90 h.p.i. with no prior signs of terminal RHD detected.

The liver of one of the RHDV2 infected rabbits was used for the production of an experimental vaccine, according to methods published previously (Lavazza and Capucci, 2016). Briefly, a 20% w/v liver homogenate (containing approximately 3 x 10^8 capsid gene copies/ml) was prepared in sterile PBS and clarified by centrifugation for 20 min at 2,000 g. Chloroform was added to the supernatant to a final concentration of 2% (v/v) and incubated at 4°C overnight, followed by a second clarification at 10,000g and 4°C. Part of the clarified supernatant was removed, mixed 1:1 with glycerol and stored at -80°C as RHDV2 inoculum for subsequent infections (0.5 ml/rabbit). Formalin was added to the remaining supernatant to a final concentration of 0.8% v/v and incubated at room temperature overnight. A second inactivation step was performed by adding formalin to a total concentration of 1% v/v with overnight incubation at 4°C. This preparation was stored at 4°C until used. Immediately prior to use, the vaccine was brought to room temperature, mixed 1:1 with Addavax (Invivogen, San Diego, USA), and between 0.6 and 1.2 ml were injected subcutaneously in the scruff of the neck.

For the production of RHDV2 ELISA antigen, a 10% w/v homogenate of the liver of the second rabbit was clarified using two centrifugation steps (20 min at 3000g, followed by 30 min at 6000g, at 4°C), and then passed through a 0.8 µm filter. The resulting homogenate was mixed 1:1 with glycerol and stored at -80°C.

A commercially produced preparation of RHDVa-K5 was used as inoculum to produce hyperimmune sera, diluted to 10,000 RID_{50}/ml (Elizabeth Macarthur Agricultural Institute, Menangle, Australia). RHDVa-Aus inoculum was prepared from a 2% of clarified liver homogenate of RHDVa-Aus (Ber-3, GenBank # KY628310) (Mahar et al., 2018b). The infectious dose of the RHDVa-Aus and the RHDV2 inoculum was not titrated.

Production of reference sera

For the production of RHDVa hyperimmune sera, five-week old rabbits were infected with RHDVa-Aus (n=4) and RHDVa-K5 (n=5). Due to the age related innate resistance to lethal RHDV infection (Matthaei et al., 2014, Neave et al., 2018) it was expected that rabbits of this age group would not succumb to fatal RHDV infection, but survive and mount a strong antibody response. Rabbits were orally infected with 0.5 ml of virus inoculum using a 1 ml syringe. Rabbits were monitored twice daily for the first four days and then daily afterwards. A small (0.1-0.2 ml) blood sample was collected from the marginal ear vein at day 0, 7 and 14. Two rabbits in each group were sacrificed and bled at 14 dpi the remaining rabbits at 20 dpi (RHDVa-K5) and 22 dpi (RHDVa-Aus) (Table 1).

Archival serum samples from five rabbits collected at various time points were used as RHDV hyperimmune reference sera. In this previous study (T. Strive, unpublished), three 5 week old New Zealand white rabbits were infected orally with 500 ID_{50} of a commercial RHDV preparation (RHDV v351, Elizabeth Macarthur Agricultural Institute, Menangle, Australia), blood samples were collected at 7 dpi and a final bleed was carried out at 14 dpi. Hyperimmune RHDV sera from an additional four rabbits harvested at various time points were available from a previous study (Matthaei et al., 2014). Seven RCV-A1 polyclonal sera were also included (Liu et al., 2012b), as well as an additional seven negative control sera.

For the production of RHDV2 polyclonal sera, rabbits needed to be partially vaccinated prior to the challenge, as young rabbits are not innately resistant to lethal RHDV2 infection (Neimanis et al., 2018a). Three five-week old rabbits and two adult rabbits of approximately three years of age were injected with the experimental vaccine preparation as described above. One adult animal was challenged 10 days after vaccination (dpv) the remaining four rabbits in this group were challenged six dpv. Monitoring and blood sampling of these animals was carried out as for the RHDVa infected rabbits (Table 1).

RHDV2 specific monoclonal antibody

A mouse monoclonal antibody (Mab) conjugated to horse radish peroxidase (4H12-HRP, lot 150416) and raised against a European strain of RHDV2 was purchased from the Instituto Zooprofilatttico Sperimentale (IZS, Brescia, Italy).

ELISA

The RHDV IgM, IgA and IgG isotype ELISA (Liu et al., 2012b), the RHDV competition ELISA (cELISA) (Cooke, 2002) and the RCV-A1 blocking ELISA (bELISA) (Liu et al., 2012a) were performed as described previously.

The RHDV2 cELISA workflow and volumes used were similar to the RHDV cELISA, with modifications. Plates were coated overnight at 4°C with a polyclonal RHDV2 rabbit serum (A#Jul, 17 dpi) at a 1:2000 dilution in carbonate buffer (pH 9.6). The plates were washed with PBS –Tween (PBS-T) prior to the addition of serial dilutions of rabbit sera starting at 1:40 dilution followed immediately with the addition of RHDV2 crude antigen at a 1:20 dilution and incubated at 37°C for 1 hour. The plate was washed and the MAb 4H12-HRP at a 1:300 dilution (0.8 µg/ml) was added and incubated at 37°C for 1 hr. The plate was washed and OPD substrate was added and incubated for 5 minutes before being stopped by the addition of 1M sulphuric acid. The plate was read at 492nM. Optimal concentrations of antigen and MAb were determined by checkerboard titrations using rabbit sera known to be positive and negative to RHDV2. Concentrations were selected that resulted in an optical density (OD) of between 1 and 1.2 for a panel of 15 negative reference sera as well as the highest signal to noise ratio when compared to the positive control serum. A serum was scored positive when the OD was reduced to 75% or less compared to the OD of the negative reference serum at the same dilution.

The IgM and IgA ELISAs were adapted to RHDV2 to assess if the sensitivity of detection of these immunoglobulins can be increased. The general set up of the assays was similar to that reported for RHDV IgM and IgA isotype ELISAs (Liu et al., 2012b), except for substituting RHDV virus like particles

with crude RHDV2 virus antigen at a 1:20 dilution, and using MAb 4H12-HRP as the detecting antibody at a 1:300 dilution (0.8 µg/ml).

RHDV2 cELISA analysis of field samples collected before and after RHDV2 arrival

A long term field site located in the central tablelands of New South Wales (Oakey Creek, 33°24'40''S, 149°22'1''E) was visited quarterly to collect serum samples from between ten and 25 healthy shot rabbits (average n=19.3), between January 2012 and January 2018. Rabbit shooting and sample collection was carried out as described previously (Cox et al., 2017), rabbit age was estimated based on dry eye lens weight as previously reported (Augusteyn, 2007). Since January 2012, samples collected for this site had been analysed using a suite of ELISAs originally developed for RHDV (Cooke, 2002).

Results:

Serum production

None of the five week old rabbits infected with RHDVa-Aus or RHDVa-K5 developed severe clinical signs or succumbed to RHD. A short fever episode of ≥40°C was observed in all four kittens infected with RHDVa-Aus and three out of four kittens infected with RHDVa-K5 between 2 and 4 dpi. These fever episodes were not associated with any changes in behavior and all animals continued to gain weight throughout the experiment.

In the group of rabbits used to produce RHDV2 polyclonal serum, no adverse reaction to the experimental vaccine was observed in any of the animals at the injection sites. No behavioral abnormalities or elevated temperatures were recorded in any of the rabbits in this group following vaccination and subsequent challenge with RHDV2.

Cross-reactivity of reference sera in RHDV isotype ELISAs

Isotype ELISAs, in particular IgM and IgA are important tools to infer disease lagovirus dynamics in rabbit populations, with IgM indicative of a recent outbreak and a boost in IgA indicating re-exposure of previously infected individuals. High levels of cross-reactivity in the RHDV IgM, IgA and IgG isotype ELISAs were observed in sera raised against RHDVa-Aus and RHDVa-K5 compared to the RHDV-Czech polyclonal sera (Table 1). Sera raised against RHDV2 also showed varying levels of cross-reactivity in this assay, but with different patterns. Cross-reactivity was highest in the IgG ELISA, with detectable titres in all animals at 8 dpi, and in one case day 6 post vaccination and prior to RHDV2 challenge. IgM antibodies were detected in all RHDV2 polyclonal reference sera although titres were lower compared to those of the RHDVa-K5, RHDV-Aus and RHDV-Czech reference sera collected at similar time points post infection. IgA antibodies are indicative of active replication of RHDV following a natural infection and are not seen in rabbits treated with inactivated vaccines (Lavazza and Capucci, 2016) and accordingly, no IgA responses were observed in this group following vaccination. IgA cross-reactivity in the RHDV2 polyclonal sera was present but with overall lower titres and with a later onset compared to rabbits infected with RHDV and RHDVa strains, indicating an active but possibly attenuated infection with RHDV2 in these previously vaccinated animals (Table 1). Overall, the IgM and IgA isotype ELISAs originally developed for RHDV appear suitable to detect disease activity of all circulating virulent strains in wild rabbit populations, although the sensitivity of detection may be slightly reduced.

Increased sensitivity and specificity in the RHDV2 adapted IgA and IgM ELISAs

When the isotype ELISAs for IgA and IgM were adapted to RHDV2 specific reagents, the titres of the RHDV2 reference sera were at least four fold greater when compared to the respective original RHDV isotype assays, and where sufficient amounts of serum were available for testing, both IgM and IgA antibodies were detected earlier (Table 1). RHDV-Czech, RHDVa-K5, and RHDVa-Aus reference sera also cross-reacted in the RHDV2 IgM and IgA ELISA, but had lower titres compared to

241 the RHDV2 reference sera (Table 1). Due to the high level of cross reactivity of RHDV2 polyclonal
242 sera in the in the IgG assay, the IgG isotype ELISA was not adapted.

243 *Cross-reactivity of reference sera in specific competition or blocking ELISAs for RCV-A1, RHDV and*
244 *RHDV2*

245 The RHDV2 cELISA developed here from Australian and European reagents proved to be highly
246 sensitive and specific. Low levels of reactivity were detected as early as 6 dpv with titres increasing
247 until the end of the trial (Table 1). Sera raised against RHDV-Czech, RHDVa-Aus and RHDVa-K5 only
248 showed low levels of cross-reactivity in this assay, only in one case exceeding titres of 1:40 (1:160,
249 K#315, 22 dpi, Table 1). None of the seven archival RCV-A1 control sera reacted in the RHDV2 cELISA.

250 In contrast, the RHDV cELISA showed low to moderate levels of cross-reactivity with RHDV2
251 polyclonal sera, and very high levels of cross-reactivity with the sera raised against the two RHDVa
252 strains (Table 1). Only one of the RHDV-Czech reference sera tested here showed a very low level of
253 cross-reactivity with the RCV-A1 bELISA (1:10, K#177, 14 dpi, Table 1). Only sera from the terminal
254 bleeds were tested in the RHDV cELISA and RCV-A1 bELISA as there was not sufficient serum left
255 from the previous sampling points.

256 *The ratio of the two specific cELISAs for RHDV and RHDV2 can be used to infer the presence of RHDV2*
257 *specific antibodies*

258 We explored if the ratios between the RHDV and RHDV2 cELISAs could be used to infer the presence
259 of RHDV2 antibodies in the populations, similar to an approach used previously to discriminate
260 between RHDV2 and European Brown Hare Syndrome Virus (EBHSV) antibodies in wild hare
261 populations in Europe (Velarde et al., 2017). While there was some level of cross-reactivity between
262 the respective cELISAs in the reference sera produced for this study, the titres were always higher
263 for the respective specific viral antigen. To investigate this further, negative results were set to a
264 titre of 1:5 for the purpose of forming ratios between reciprocal titres, and only samples were

included in the analysis that had returned a positive reaction in either of the two assays, as described previously (Velarde et al., 2017). Due to the high likelihood of cross reactivity at very low dilutions, only test results > 1:40 were considered for this analysis. The RHDV2 cELISA/RHDV cELISA titre ratios were >1 in all reference sera raised against RHDV2, with ratios ranging from 1 to 100. In all reference sera raised against RHDV or RHDVa the ratios were <1, ranging from 0.008 to 0.125 for sera raised against RHDVa-K5 and RHDVa-Aus, and from 0.0002 to 0.0156 for sera raised against RHDV-Czech.

We then applied this method to retrospectively test archival serum samples collected at the Oakey Creek long term study site where these were available. Here, approximately 20 rabbits had been sampled quarterly since January 2012 and analysed with the serological assays originally developed for RHDV (Cooke et al., 2000). RHDV IgM and IgA assays were only carried out at a 1:40 dilution to detect presence or absence of recent disease activity but were not titrated. These samples were re-tested in the RHDV2 cELISA described here to determine the time of RHDV2 arrival at this site. Initially we analysed a very early sample of n=20 rabbits from autumn 2012 as a negative baseline, as it was considered very unlikely that RHDV2 would have been present in Australia over three years prior to its detection. We then continued the analysis starting with the most recent samples, working backwards until four consecutive samples showed no serological evidence of RHDV2. Unexpectedly, serological analysis revealed clear evidence for presence of RHDV2 antibodies in this population with several samples resulting in an RHDV2cELISA/RHDVcELISA ratio of >1 as early as January 2015 (Figure 1). This indicates that RHDV2 must have arrived at this site sometime between the sampling periods October 2014 and January 2015, which pre-dates the first case report of RHDV2 in a deceased rabbit by at least six months (Hall et al., 2015). While fluctuating, overall seroprevalence to RHDV2 increased in later sampling periods coinciding with an overall decrease of animals classified as positive to RHDV.

289 Some level of cross reactivity was observed in the RHDV2 cELISA prior to RHDV2 arrival. Four out of
290 96 samples analysed that were collected prior to January 2015 showed low level cross reactivity in
291 the RHDV2 cELISA with titres between 1:40 and 1:80. However in all these cases the individual
292 rabbits had higher titres in the RHDV cELISA resulting in a ratio of <1 in all cases.

293 IgM antibodies indicative of recent lagovirus outbreaks were frequently detected in this population
294 prior to the arrival of RHDV2, namely in winter and spring 2012, summer 2014, and winter and
295 spring 2014. No evidence for recent virus activity was detected in the following five sampling
296 periods, but then again in autumn and spring 2016, and again in autumn 2017 (Figure 1). In order to
297 ascertain which virus was likely causing these outbreaks, we investigated the antibody profiles in the
298 recent cohorts of rabbits (Figure 2). Although some rabbit breeding in this part of Australia is
299 possible year round, the main breeding events occur in winter/spring. Accordingly, members of each
300 new cohort of rabbits starts to appear in the summer shot sample, and subsequent sampling periods
301 throughout the year reflect the increase in age of this cohort (Figure 2). As expected, all animals
302 scored as positive to RHDV with a cELISA ratio <1 prior to January 2015. After the arrival of RHDV2 at
303 this site, in start to appear in the January 2015 sample. However, evidence for RHDV positive animals
304 in less than 12 months of age was found in January 2017 and October 2017. These animals had not
305 yet been born at the time of RHDV2 arrival, indicating that both RHDV and RHDV2 must have been
306 involved in the three periods of virus activity recorded since RHDV2 arrival at this site. The RHDV
307 cELISA titres in these young rabbits were high, ranging from 1:80 to 1:2560 (1:840 avg), indicating a
308 strong immune response from an active RHDV infection rather than the presence of residual
309 maternal antibodies.

310 **Discussion:**

311 Specific serology of different lagoviruses can be challenging, as high levels of cross-reactivity are
312 often observed even between antigenically very different members of this genus (Capucci et al.,
313 1996b, Liu et al., 2012b, Nagesha et al., 2000). For the more specific detection of different viruses,

less sensitive and more specific cELISAs or bELISAs have been described (Capucci et al., 1991, Collins et al., 1995, Liu et al., 2012a), however, isotype ELISAs detecting IgG antibodies are so cross-reactive that they have historically been used to infer the presence of previously unknown and often antigenically quite different lagoviruses (Capucci et al., 1996b, Cooke et al., 2000, Nagesha et al., 2000, Robinson et al., 2002).

These difficulties notwithstanding, there is a need for improved serological tools to discriminate between antibodies to the different lagoviruses circulating in Australia, to better understand their role in naturally occurring disease dynamics as well as biological control operations of wild rabbits. For over two decades only RHDV and RCV-A1 were known to be present in Australia, and their disease dynamics have been studied extensively, utilising specific cELISAs or bELISAs for RHDV and RCV-A1 as well as isotype ELISAs for both viruses (Cooke et al., 2000, Liu et al., 2012a, Liu et al., 2012b). The recent arrival of two different strains of RHDVa (RHDVa-Aus and RHDVa-K5) as well as RHDV2 has highlighted the need to update and extend the panel of serological tools available on this continent to enable better studies of the epidemiology and interactions of these viruses.

Disease dynamics of RHDV can be inferred by interpreting the reaction profiles in different isotype and cELISAs (Cooke, 2002). In this approach, IgM is interpreted as an indicator of a recent outbreak and first time exposure of an individual to RHDV, a boost in IgA titres as a measure for re-exposure to RHDV, and the highly sensitive but cross-reactive IgG is used to infer the presence of maternal antibodies in very young rabbits. In our study, the reference sera raised against RHDV-Czech, RHDVa-Aus, RHDVa-K5 and RHDV2 all reacted to varying degrees in these assays, indicating that they should be suitable to detect broad patterns of disease activity caused by any of the virulent lagovirus strains in wild rabbit populations in Australia, however they do not allow for the discrimination between the various strains.

Adaptation of the IgM and IgA ELISA to RHDV2 by using RHDV2-specific antigen and Mab increased the sensitivity of these assays for RHDV2 IgM and IgA detection substantially. While the use of the

339 original RHDV IgA and IgM assays is sufficient to infer broader disease activity patterns in wild rabbit
340 populations for all lagoviruses including RHDV2 (Figure 1), switching to these more sensitive assays
341 for future large scale field epidemiology studies should be considered if RHDV2 remains the
342 dominant strain in the Australian landscape (Mahar et al., 2018a).

343 The RHDV2 cELISA described here proved to be highly specific for the detection of RHDV2
344 antibodies, with low levels of cross-reactivity to the experimentally produced RHDV and RHDVa
345 reference sera as well as the sera from wild rabbits collected before the arrival of RHDV2 in
346 Australia. In contrast, the existing RHDV cELISA (Capucci et al., 1991) showed low to medium levels
347 of cross-reactivity to RHDV2 and high levels of cross-reactivity to both RHDVa-Aus and RHDVa-K5.
348 However, in the RHDV and RHDV2 cELISAs, titres were higher in the sera raised against the
349 respective strain, such that the ratios of the reciprocal titres of the two assays can be used to infer
350 which strain the rabbit was most likely exposed to. It needs to be noted that this method classifies
351 rabbits into positive to either RHDV or RHDV2, and therefore does not allow for the detection of
352 mixed infections and the true prevalence to both RHDV and RHDV2 is likely underestimated.
353 However the method does allow to detect presence of RHDV2 and should allow to discern broader
354 trends within rabbit populations.

355 When applied to a sample collection from a long term field monitoring site, this method showed
356 clear evidence for RHDV2 activity in this population at least six months prior to the first RHDV2 case
357 reported in Australia (Hall et al., 2015), and confirms inferences made from phylogenetic analyses of
358 viral sequences suggesting that RHDV2 had circulated in Australia several months prior to its first
359 detection in May 2015 (Mahar et al., 2018a).

360 The inclusion of age data into the analysis allowed the confirmation of RHDV activity in this
361 population after July 2016. In addition, including age data into the analysis may provide a more
362 accurate estimate of disease dynamics than analysing overall seroprevalence data alone. A large
363 proportion of rabbits present in every shot sample were >500 days old, and may therefore confound

the analysis of recent virus activity. In addition, if some level of immunological cross protection exists between RHDV and RHDV2 (Calvete et al., 2018), the removal of susceptible rabbits by an RHDV2 outbreak from the population could result in an apparent increase of RHDV seroprevalence, due to the resulting increased proportion of surviving old RHDV seropositive animals in the sample.

Conclusions:

The analysis of the reference sera as well as field sera collected pre- and post-RHDV2 arrival in Australia indicates that existing RHDV IgA and IgM isotype ELISAs are suitable to infer disease dynamics of all virulent RHDV strains currently circulating in Australia. The ratio between the RHDV2 and RHDV cELISA titres allows for the detection of the presence of RHDV2 specific antibodies with high levels of certainty, even in the presence of RHDV or RHDVa antibodies. In combination, the methods described here should allow for retrospective analysis of archival field sera to study the spread and impact of RHDV2 on Australian rabbit populations as well as allow for a more accurate estimate of the time of RHDV2 arrival at various site in Australia. Including rabbit age data, where available, will improve these analyses. However, due to the high levels of cross-reactivity of RHDV2 reference sera in the RHDV cELISA, detecting RHDV or RHDVa antibodies in samples collected post RHDV2 arrival is challenging. In particular, inferring mixed infections of RHDV/RHDVa/RHDV2 or confirming the absence of RHDV antibodies in RHDV2 positive populations is difficult to discern. Similarly, distinguishing between antibodies to RHDV and RHDVa strains is not feasible with the serological tools currently available. Despite these remaining challenges in the differential serodiagnostics of Australian lagoviruses, the additional assays described here represent an important addition to the tool kit that will benefit ongoing continent-wide lagovirus epidemiology studies.

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558

559 **Tables**

560 **Table 1:** Serological reaction profiles in experimentally generated polyclonal sera against four types

561 of RHDV and RCV-A1.

562

rabbit ID	dpv	dpi	RHDV IgM	RHDV IgA	RHDV IgG	RHDV cELISA	RHDV-2 IgM	RHDV-2 IgA	RHDV-2 cELISA	RCV-A1 bELISA
RHDV-2 polyclonal sera										
A#Zor	0		0	0	0	–	–	–	0	–
A#Zor	6		640	0	160	–	40960	0	160	–
A#Zor	10	0	640	0	160	–	40960	0	320	–
A#Zor	14	4	640	0	640	–	40960	0	160	–
A#Zor	16	6	2560	0	2560	–	40960	10240	1280	–
A#Zor	24	14	640	80	2560	–	10240	10240	2560	–
A#Zor	26	16	640	80	10240	–	10240	10240	2560	–
A#Zor	34	24	640	640	10240	320	2560	40960	5120	0
A#Jul	0		0	0	0	–	–	–	0	–
A#Jul	5		0	0	0	–	640	0	10	–
A#Jul	6	0	0	0	0	–	2560	0	80	–
A#Jul	14	8	0	20	2560	–	10240	640	320	–
A#Jul	16	10	0	0	10240	–	10240	640	1280	–
A#Jul	24	17	80	20	10240	20	10240	10240	1280	0
K#316	0		0	0	0	–	–	–	0	–
K#316	5		0	0	0	–	–	–	0	–
K#316	6	0	0	0	0	–	640	–	10	–
K#316	14	8	640	160	2560	–	40960	10240	1280	–
K#316	16	10	640	160	10240	–	40960	10240	160	–
K#316	24	17	640	40	10240	20	10240	2560	2560	0
K#317	0		0	0	0	–	–	–	0	–
K#317	5		0	0	0	–	–	–	0	–
K#317	6	0	160	0	0	–	–	–	10	–
K#317	14	8	640	80	2560	–	10240	10240	160	–
K#317	16	10	160	80	2560	–	10240	10240	160	–
K#317	24	17	320	320	2560	20	10240	2560	1280	0
K#318	0		0	0	0	–	–	–	0	–
K#318	5		0	0	0	–	–	–	10	–
K#318	6	0	0	0	0	–	–	–	20	–
K#318	14	8	160	80	2560	–	–	–	320	–
K#318	16	10	160	80	10240	–	10240	10240	320	–
K#318	24	17	640	80	10240	20	10240	2560	1280	0
RHDVa-K5 polyclonal sera										
K#307		0	0	0	0	–	–	–	0	–
K#307		7	40960	2560	2560	–	10240	2560	10	–

K#307	14	10240	2560	2560	–	2560	640	0	–
K#307	20	10240	10240	40960	320	640	2560	10	0
K#308	0	0	0	0	–	–	–	0	–
K#308	7	10240	1280	2560	–	10240	640	0	–
K#308	14	10240	1280	2560	–	2560	640	10	–
K#308	20	5120	2560	10240	320	640	2560	40	0
K#309	0	0	0	0	–	–	–	0	–
K#309	7	40960	2560	2560	–	10240	2560	10	–
K#309	14	10240	1280	2560	–	640	160	10	–
K#309	20	5120	1280	10240	640	640	640	40	0
K#310	0	0	0	0	–	–	–	0	–
K#310	7	40960	2560	2560	–	2560	160	10	–
K#310	14	10240	640	2560	320	640	640	10	0
K#311	0	0	0	0	–	–	–	0	–
K#311	7	40960	2560	2560	–	10240	160	20	–
K#311	14	2560	5120	2560	640	2560	2560	10	0
	RHDVa-Aus polyclonal sera								
K#312	0	0	0	0	–	–	–	0	–
K#312	7	40960	640	10240	–	–	–	0	–
K#312	14	10240	2560	10240	640	10240	2560	10	0
K#313	0	0	0	0	–	–	–	0	–
K#313	7	40960	640	2560	–	2560	40	10	–
K#313	14	10240	1280	2560	320	2560	640	0	0
K#314	0	0	0	0	–	–	–	0	–
K#314	7	10240	2560	10240	–	640	40	0	–
K#314	14	–	2560	40960	–	–	–	0	–
K#314	22	10240	5120	40960	1280	2560	640	10	0
K#315	0	0	0	0	–	–	–	0	–
K#315	7	40960	10240	40960	–	10240	160	0	–
K#315	14	10240	2560	40960	–	2560	160	10	–
K#315	22	10240	1280	10240	640	2560	640	160	0
	RHDV-Czech polyclonal sera								
K#175	4	640	0	0	–	–	–	0	0
K#175	7	40960	2560	40	–	640	0	0	0
K#175	14	40960	10240	40960	5120	160	1280	0	0
K#176	4	160	0	0	–	–	0	0	0
K#176	7	10240	1280	640	–	640	160	0	0
K#176	14	20480	10240	10240	320	40	160	0	0
K#177	4	2560	0	0	–	–	–	0	0
K#177	7	10240	160	2560	–	2560	160	0	0
K#177	14	40960	5120	10240	5120	2560	640	10	10
K#184	4	2560	0	160	–	0	0	0	0
K#185	4	80	0	0	–	0	0	0	0
K#135	0	0	0	0	–	–	–	0	–
K#135	15	40960	10240	40960	10240	640	160	0	0
K#136	0	0	0	0	–	–	–	0	–
K#136	15	40960	10240	40960	10240	640	640	10	0

K#137	0	0	0	0	–	–	–	D	–
K#137	4	40960	5120	0	–	2560	–	10	0
K#138	0	0	0	0	–	–	–	0	–
K#138	15	40960	40960	40960	20480	2560	640	0	0
K#139	0	0	0	0	–	–	–	D	–
K#139	15	10240	5120	40960	5120	0	0	0	0
RCV-A1 polyclonal sera									
RCV-3	7	0†	0†	0†	0†	–	–	0	160††
RCV-4	7	2560†	320†	320†	80†	–	–	0	320††
RCV-8	11	0†	0†	40†	0†	–	–	0	40††
RCV-11	28	0†	160†	2560†	0†	–	–	0	160††
RCV-12	28	0†	0†	640†	0†	–	–	0	160††
RCV-24	9	0†	0†	0†	0†	–	–	0	320††
RCV-25	9	160†	0†	320†	0†	–	–	0	160††

dpv = Days post vaccination
dpi = Days post infection
† Data from Liu et al., 2012a
††Data from Liu et al., 2012b
D= doubtful

Figures

Figure 1: Calicivirus disease activity and proposed arrival of RHDV2 inferred from serological data at a long term monitoring site in NSW (Oakey Creek). A. Sera from between 10 and 25 (avg 19.3) healthy shot rabbits were analysed for RHDV IgM and IgA antibodies four times per year. Presence of IgM antibodies and/or an increase in IgA prevalence is used to infer recent virus activity (Cooke et al., 2000). Rabbits were scored positive to RHDV2 when the ratio of the RHDV2-cELISA/RHDV-cELISA titres was >1 and positive to RHDV when the ratio was <1. Prior to January 2015, RHDV prevalence was inferred as the proportion of rabbits positive to the RHDVcELISA. Black arrows indicate inferred virus outbreaks or prolonged periods of virus activity. The vertical dashed grey line indicates the time RHDV2 was first reported in Australia (Hall et al., 2015). The vertical red box indicates the

578 approximate proposed arrival period of RHDV2 at this site. B. Individual rabbits classified as positive

579 to RHDV or RHDV2 according to their age. Age in days was determined using the dry eye lens weight

580 according to previously described methods (Augusteyn, 2007).

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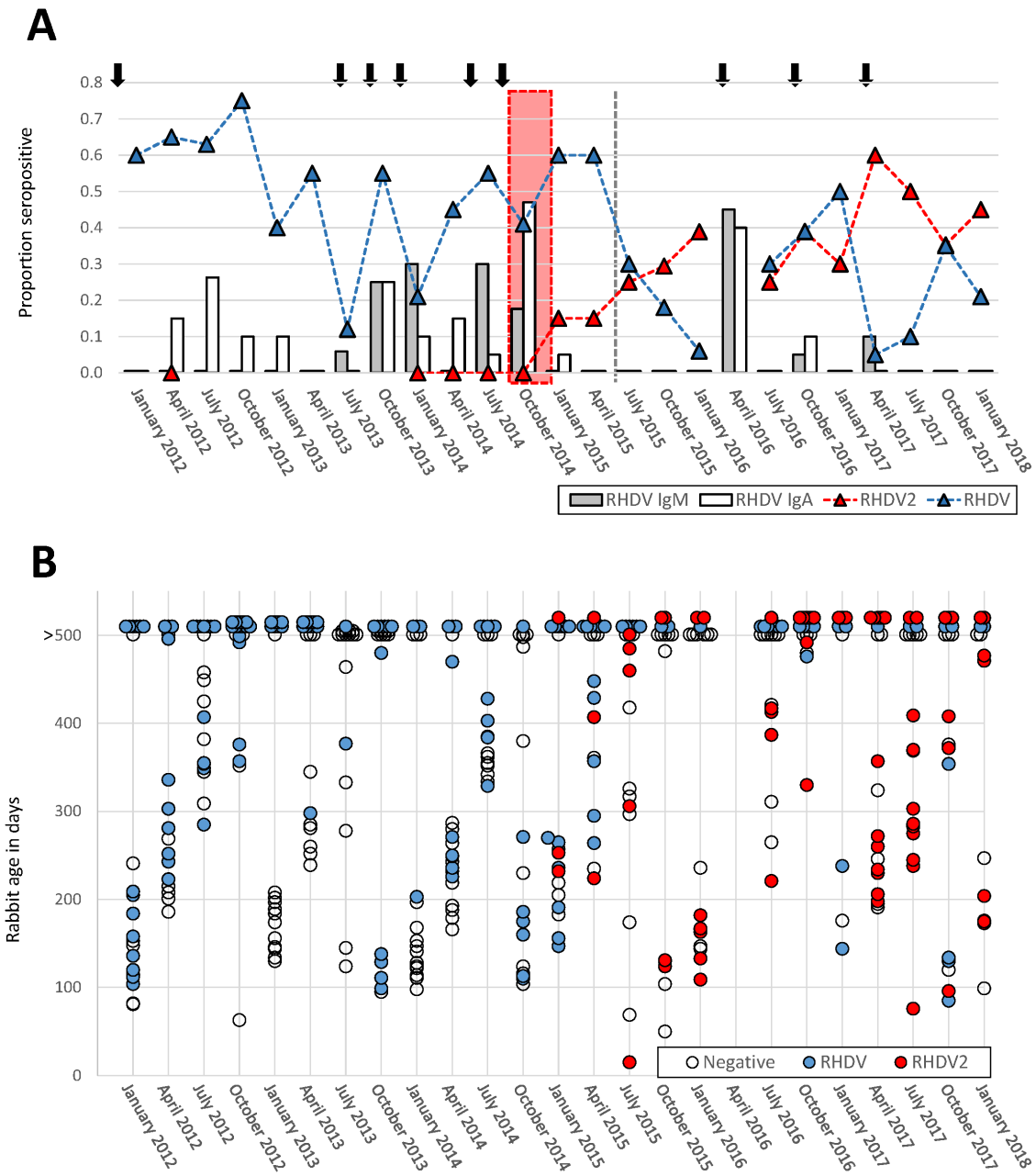

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