bioRxiv preprint doi: https://doi.org/10.1101/585885; this version posted March 25, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

1 **Carrion flies as sentinels for monitoring rabbit calicivirus activity in Australia**

2 Running title: Rabbit calicivirus detection in blowflies

3 **Robyn N Hall[1,2]*, Nina Huang[1], John Roberts[3], Tanja Strive[1,2]**

4 1. CSIRO Health & Biosecurity, Canberra, Australia

5 2. Centre for Invasive Species Solutions, Canberra, Australia

6 3. CSIRO Land and Water, Canberra, Australia

7

8 **Summary**

9 Rabbit caliciviruses are an essential tool for managing wild rabbit populations in Australia.
10 Our understanding of rabbit calicivirus epidemiology in Australia currently depends on
11 members of the public submitting liver samples from dead rabbits through a monitoring
12 program called Rabbitscan. However, many wild rabbits die in inaccessible locations or are
13 scavenged before sampling can occur, leading to considerable sampling bias. In this study
14 we screened field-caught carrion flies for the presence of rabbit caliciviruses to monitor virus
15 circulation patterns in the landscape, with an aim to establish a less biased epidemiological
16 surveillance tool. Carrion flies were collected from two study sites over a 22 month period
17 and these samples were used to optimise and validate molecular testing methods in this
18 sample type for the currently circulating rabbit calicivirus variants. Virus was clearly
19 detectable in field-caught carrion flies using optimised SYBR-green RT-qPCR and RT-PCR
20 assays. However, variant identification was frequently hindered by the low virus loads
21 present in carrion fly samples and spurious RT-PCR amplification. This was overcome by
22 frequent sampling, which effectively acts as replicate sampling to verify inconclusive results.
23 There was good correlation between virus detections in carrion flies and in samples
24 recovered from wild rabbits, both temporally and for virus variant identification. The methods
25 reported here provide a robust and efficient additional surveillance tool to monitor rabbit
26 calicivirus activity at a landscape scale, which in turn can help to guide more effective rabbit
27 management programs.

28

bioRxiv preprint doi: https://doi.org/10.1101/585885; this version posted March 25, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

29 Keywords: calicivirus; RHDV; blowflies; surveillance

30

31 **Introduction**

32 Wild European rabbits (*Oryctolagus cuniculus*) are one of Australia's most invasive
33 vertebrate pests, directly threatening over 300 native plant and animal species and costing
34 Australian agriculture over $200 million AUD annually. Wild rabbits are currently controlled
35 using an integrated approach involving both conventional pest management methods, such
36 as shooting and poisoning, and biocontrol agents, principally rabbit caliciviruses (Le Pendu
37 et al., 2017). It is feasible that synergism between virus variants could be exploited to
38 maximise the effectiveness of biocontrol programs, however this requires knowledge of prior
39 infection history in different wild rabbit populations. Five pathogenic rabbit caliciviruses have
40 thus far been reported in Australia: 1) the original V-351 RHDV (GI.1c) deliberately released
41 as a biocontrol agent in the mid-1990s, and its descendants; 2) an additional rabbit
42 biocontrol agent and released nationwide in March 2017 (Invasive Animals CRC, 2014); 3) a
43 GI.1a variant, later identified as a recombinant GI.4e-GI.1a (subsequently referred to as
44 GI.1a-Aus) (Mahar et al., 2018); 4) a recombinant GI.1b-GI.2 virus (also known as RHDV2 or
45 RHDVb) (Hall et al., 2015); and 5) a recombinant GI.4e-GI.2 virus, comprising the non-
46 structural genes of GI.1a-Aus and the structural genes of GI.2 (Hall et al., 2018).
47 Both molecular and serological methods have previously been used to monitor rabbit
48 calicivirus activity across Australia. Molecular methods are relatively quick, accurate, and
49 sensitive, however, sample collection is extremely biased, with most wild rabbits dying in
50 inaccessible locations and/or being removed by scavengers before collection. Serological
51 testing works well as a population tool to identify previous exposure of populations to rabbit
52 caliciviruses (Cooke et al., 2000; Cox et al., 2017). However, the confidence and power of
53 the conclusions drawn from results of serological testing depends strongly on the sample
54 size, and collection of shot samples from multiple animals requires considerable time and
55 person resources. Furthermore, serological tools are only able to broadly distinguish
56 between GI.1 and GI.2 viruses, and accurate identification to the variant level and of mixed

bioRxiv preprint doi: https://doi.org/10.1101/585885; this version posted March 25, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

57 infections is challenging due to cross-reactivity between assays (Strive et al, in preparation).
58 Due to the limitations of existing methods, a less biased and more systematic sampling
59 method is required for surveillance of rabbit calicivirus activity across Australia, firstly to
60 determine the spatial and temporal distribution of specific virus variants and subsequently to
61 infer the interactions between the different viruses.

62 Carrion flies have long been known to be able to mechanically transmit rabbit
63 caliciviruses (Asgari et al., 1998; Cooke and Fenner, 2002; McColl et al., 2002; Henning et
64 al., 2005; Schwensow et al., 2014). Laboratory studies have detected rabbit calicivirus
65 (GI.1c) by RT-PCR in various fly species (*Calliphora*, *Chrysomya*, *Hydrotaea*, *Lucilia*, *Musca*,
66 *Sarcophaga,* and *Oxysarcodextia* species), as well as in two species of *Aedes* mosquitos
67 (Asgari et al., 1998; McColl et al., 2002; Henning et al., 2005). It was further shown that virus
68 was detectable in flies for more than 11 days, however, virus was only detectable on the legs
69 of flies for 7 hours (Asgari et al., 1998). Virus has also been detected in flyspots (faecal and
70 regurgitation spots) by RT-PCR, and it has been demonstrated that flyspots contained
71 sufficient virus to cause disease when used to infect susceptible rabbits by oral infection
72 (Asgari et al., 1998). Transmission trials have also shown that bush flies (*Musca*
73 *vetustissima*) fed on carcasses of GI.1c-infected rabbits were able to naturally transmit
74 infection to susceptible rabbits (McColl et al., 2002).

75 Since carrion flies are relatively easy and inexpensive to collect, they could prove a
76 valuable additional systematic sampling tool for landscape scale monitoring of RHDV activity
77 using high-sensitivity molecular detection methods. As the genetic diversity of rabbit
78 caliciviruses has greatly increased in Australia in recent years, and several virus variants
79 may co-circulate at the same time, the aim of this study was to develop molecular tools and
80 protocols to reliably detect circulating rabbit caliciviruses in carrion flies to the virus variant
81 level. These tools were subsequently validated by analysing carrion flies sampled at two
82 sites closely monitored for rabbit calicivirus activity over a period of 22 months.
83

84 **Materials and methods**

bioRxiv preprint doi: https://doi.org/10.1101/585885; this version posted March 25, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

85 *Fly collection*

86 Carrion flies were collected approximately weekly between 26 September 2016 and 30
87 June 2018 at two locations, Murrumbateman NSW (-35.00, 149.02) and Black Mountain
88 ACT (-35.27, 149.11). Flies were trapped initially in LuciLure traps (BioGlobal, Australia) with
89 commercial attractant and later, for convenience, in Envirosafe™ fly traps (Bunnings,
90 Australia) baited with either Envirosafe™ fly attractant (Bunnings, Australia), chicken liver, or
91 commercial attractant (BioGlobal, Australia), or a combination of the above. Attractant was
92 placed in specimen jars covered with a gauze swab to prevent flies coming into direct
93 contact with the bait (Figure 1). Traps were left out for one to seven days and upon collection
94 were briefly frozen at -20 °C to immobilise flies before flies were transferred to storage
95 containers at -20 °C. Fly traps and all components (except bait) were soaked in 10%
96 household bleach for 30 minutes before being rinsed and re-used.

97 *Optimisation of pooling strategy*

98 To optimise the pooling strategy (number and species of flies per pool) an early field
99 collection (Black Mountain 25/11/2016) that contained a large number of flies and was
100 positive for GI.4e-GI.2 rabbit calicivirus was used. We elected not to use laboratory-spiked
101 samples since this field collection more accurately represents the characteristics of our final
102 target collections.

103 To determine the effect of fly species on viral load and analytical sensitivity, four to five
104 flies of five different species (based on morphological identification) were examined
105 individually for the presence of virus. RNA was extracted and RT-qPCR and RT-PCR were
106 conducted as described below. Species was confirmed by amplifying a region of
107 mitochondrial DNA using the primers C1-J-2495 and C1-N-2800 (Wells and Sperling, 2001)
108 and Platinum Taq DNA polymerase (Life Technologies) as per manufacturer's instructions.
109 Sanger sequencing was then conducted at the Australian Cancer Research Foundation
110 Biomolecular Resource Facility, and sequences were analysed for the closest genetic match
111 using NCBI Blastn against the nr database as implemented in Geneious 11.1.

bioRxiv preprint doi: https://doi.org/10.1101/585885; this version posted March 25, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

112 To determine the effect of fly number on analytical sensitivity, we prepared five
113 replicate pools containing either one, three, five, or ten flies (mixed species) per pool. RNA
114 was extracted and RT-qPCR and RT-PCR were conducted as described below.

115 *Optimisation of RNA extraction method*

116 To determine the effect of alternative homogenisation buffers and pre-extraction
117 freeze-drying on analytical sensitivity, we prepared 12 replicate pools containing ten flies
118 (mixed species) per pool. RNA was extracted from pools as follows: three pools using the
119 standard protocol described below; three pools using a modified protocol incorporating
120 freeze-drying of flies; three pools using the standard protocol with an alternative 10 mM Tris-
121 HCl pH 8.5 homogenisation buffer containing 10 μl.ml^{-1} β-mercaptoethanol; and three pools
122 using the standard protocol with an alternative phosphate buffered saline (PBS)
123 homogenisation buffer containing 10 μl.ml^{-1} β-mercaptoethanol.

124 RNA was extracted using the Maxwell RSC simplyRNA tissue kit (Promega,
125 Alexandria, NSW) on a Maxwell RSC system (Promega). Briefly, to each sample 10 volumes
126 (minimum 200 μl) of homogenisation buffer was added per mg of fly weight. Samples were
127 heated at 70 °C for 2 minutes before homogenisation using a Precellys 24-dual tissue
128 homogeniser (Bertin Technologies, Montigny-le-Bretonneux, France). Samples were clarified
129 at 3,000 *g* for 3 minutes and homogenate (minimum 200 μl) was combined with an equal
130 volume of lysis buffer and mixed by vortexing. This homogenate/lysis buffer mix (400 μl) was
131 used for extraction. The complete protocol is available at
132 dx.doi.org/10.17504/protocols.io.ux7exrn. Known rabbit calicivirus negative flies (*Calliphora*
133 *augur* reared in clean conditions in the laboratory) were extracted in parallel with each set of
134 RNA extractions as a negative extraction control.

135 This protocol was modified as follows when flies were freeze-dried. Samples were
136 freeze-dried overnight in a Flexi-Dry MP freeze-dryer (FTS Systems, Stone Ridge, New
137 York). Once dry, samples were homogenised using a Precellys 24-dual tissue homogeniser
138 (Bertin Technologies). Ten volumes of homogenisation buffer was then added per mg of dry
139 fly tissue. Samples were heated, clarified, and mixed with lysis buffer as described above.

bioRxiv preprint doi: https://doi.org/10.1101/585885; this version posted March 25, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

140 All RNAs were stored at -80 °C.

141 *RT-qPCR and endpoint strain-specific PCRs*

142 To compare analytical sensitivity during validation, viral loads were quantified using a
143 SYBR-green-based RT-qPCR for the generic detection of all Australian rabbit caliciviruses,
144 as described previously (Hall et al., 2018). Detections were confirmed using the strain-
145 specific lagovirus multiplex RT-PCR assay described previously (Hall et al., 2018). RNAs
146 were diluted 1/10 in nuclease-free water prior to RT-PCR and were used undiluted as
147 template in the RT-qPCR assay.

148 For routine monitoring of field-caught fly samples, a serial testing procedure was
149 used where samples were first run through the universal RT-qPCR assay and those samples
150 with virus loads greater than 1000 capsid gene copies per µl of RNA (cut-off derived during
151 validation) were subsequently analysed using the strain-specific RT-PCR assays in
152 singleplex format for virus variant identification.

153 *Strain identification in rabbits and hares*

154 Liver samples from rabbits and hares found dead at Black Mountain or
155 Murrumbateman were screened for rabbit caliciviruses using previously reported methods
156 (Hall et al., 2018). No animal ethics permit is required in Australia for sample collection from
157 rabbits or hares that are found dead.

158

159 **Results**

160 A series of experiments was conducted to validate and optimise molecular testing
161 protocols for the strain-specific detection of rabbit caliciviruses in carrion flies. Specifically,
162 we focussed on optimising the pooling strategy (number and species of flies from individual
163 traps) and RNA extraction method using flies from a single known-positive field collection.
164 We then assessed the suitability of existing RT-qPCR and RT-PCR assays for detection of
165 rabbit caliciviruses in fly RNA. These existing assays were developed for use on liver RNA,
166 although other sample types including bone marrow, whole blood, maggots, and skin have
167 also been used for testing and returned positive results (unpublished data). Once an optimal

bioRxiv preprint doi: https://doi.org/10.1101/585885; this version posted March 25, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

168 test procedure was determined, we used these protocols to monitor rabbit calicivirus
169 circulation in the environment using carrion flies over a 22 month period at two locations,
170 Murrumbateman (NSW) and Black Mountain (ACT), approximately 35 km apart.

171 ***Species or number of flies does not affect sensitivity of detection***

172 Five different fly species were tested to determine whether virus load correlated with
173 species. Individuals were identified morphologically as either *Calliphora augur*, *C.*
174 *canimicans bezzii*, *C. stygia, Chrysomya rufifacies*, or *Chrysomya varipes* (Wallman, 2001).
175 A region of the mitochondrial DNA of these individuals was also sequenced to verify species
176 identity. Species was confirmed in most cases, with a few exceptions. One individual
177 morphologically identified as *C. stygia* was genetically classified as *C. hilli hilli* and all *C.*
178 *canimicans bezzii* were genetically identified as *C. ochracea*. Morphologically, *C. ochracea* is
179 quite distinctive and further investigation revealed that no sequences for *C. canimicans*
180 *bezzii* are present in GenBank. Therefore, this classification of *C. ochracea* is likely to be
181 incorrect. These individuals were subsequently referred to as *Calliphora* (species
182 unidentified).

183 Every individual fly apart from one *Chrysomya rufifacies* was positive on both RT-
184 qPCR and strain-specific RT-PCR (Figure 2a). There was no significant difference in the
185 viral load carried by different fly species. To test whether the number of flies used per RNA
186 extraction affected the sensitivity of detection five replicate pools of either three, five, or ten
187 individual flies of mixed species were examined by RT-qPCR. Again, no significant
188 differences in viral loads were observed in the different pool sizes, and the viral loads in
189 pooled samples were comparable to those detected in individual flies (Figure 2b).

190 ***Optimisation of RNA extraction protocols for rabbit calicivirus detection in***
191 ***carrion flies***

192 When processing larger pools of flies it became apparent that for increased
193 throughput an alternative homogenisation buffer would be required, since the volume of
194 commercial homogenisation buffer provided in the kit was insufficient. We therefore
195 compared two alternative homogenisation solutions, either 10 mM Tris-HCl pH 8.5 or 1x

bioRxiv preprint doi: https://doi.org/10.1101/585885; this version posted March 25, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

196 PBS, both containing 10 µl.ml^{-1} β-mercaptoethanol to inactivate environmental RNases, with
197 the commercial buffer. We also assessed the suitability of freeze-drying flies prior to RNA
198 extraction, in order to quantitate virus load on a dry matter basis. No significant differences in
199 viral load were observed between freeze-dried or fresh flies or between the alternative
200 homogenisation buffers used (Figure 3).

201 ***Modification of existing RT-qPCR and RT-PCR assays enabled robust detection***
202 ***of rabbit caliciviruses in carrion flies to the virus variant level***

203 An optimised RNA extraction protocol that included freeze-drying of flies was then
204 used to process 99 fly pools collected between September 2016 and June 2018 at two
205 locations near Canberra ACT. These were screened in series as previously recommended
206 (R. N. Hall et al., 2018)—initially samples were tested in a universal lagovirus SYBR-green-
207 based RT-qPCR and those above the limit of quantification of the assay (i.e. 100 capsid
208 copies per µl of RNA) were subtyped using a strain-specific RT-PCR. Of the 99 pools
209 collected over the sampling period, 49 (49%) samples had viral loads above this limit of
210 quantification. When using the RT-PCR in multiplex format, frequently non-specific bands
211 were present that made interpretation very challenging, despite diluting the RNA template to
212 mitigate the effects of PCR inhibitors that may be present. To try to avoid the formation of
213 these non-specific bands, subtyping was performed using singleplex RT-PCR assays for
214 each virus variant (i.e. GI.1, GI.2, GI.1a-K5, GI.1a-Aus). Based on these singleplex endpoint
215 RT-PCR results, a virus strain was unambiguously assigned for 24 of these 49 samples
216 (49%) (Figure 4). The remaining 25 samples continued to return inconclusive results, either
217 returning negative results on RT-PCR despite being positive on RT-qPCR, inconsistent
218 results on repeated testing, or only amplifying very weak bands. Contrastingly, on rabbit liver
219 samples strain determination is extremely accurate on samples with viral loads greater than
220 100 capsid copies per µl of RNA (R. N. Hall et al., 2018). Increasing the RT-qPCR cut-off to
221 1000 capsid copies per µl of RNA increased the stringency of testing, with 26 samples
222 testing positive. Of these samples, strain was unambiguously determined in 18 (69%) of
223 those samples.

bioRxiv preprint doi: https://doi.org/10.1101/585885; this version posted March 25, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

224 ***Carrion flies provide a cheap and efficient method to monitor rabbit calicivirus***
225 ***outbreaks***

226 Using the modified RT-qPCR threshold value of 1000 capsid copies per µl of RNA,
227 viral loads were plotted as a time series to determine whether carrion flies were suitable
228 proxies for detecting rabbit calicivirus circulation in the environment. Distinct calicivirus-
229 positive fly collections were observed over the sampling period based on this fly sampling
230 method (Figure 5). Positive samples were observed at Black Mountain in December 2016,
231 April 2017, October 2017, December 2017-January 2018, and April 2018. Similarly, positive
232 fly samples were observed at Murrumbateman in April 2017, October 2017, January-
233 February 2018, and March-April 2018. Murrumbateman potentially also had a positive
234 sample in December 2016, however, the virus variant could not be determined conclusively.
235 At both locations, calicivirus circulation detected via fly sampling correlated closely with
236 detection of calicivirus-positive dead lagomorphs (i.e. rabbits and hares), both temporally
237 and for virus variant identification (Figure 5). At Black Mountain calicivirus-positive
238 lagomorphs were detected in April-May 2017, October 2017, January 2018, and May-June
239 2018, while at Murrumbateman they were detected in April-June 2017, October-November
240 2017, and June 2018.
241
242 **Discussion**

243 Monitoring rabbit calicivirus circulation across Australia is important both to inform
244 management of wild rabbit populations and control and prevention of calicivirus outbreaks in
245 domestic rabbits. Knowing the geographical and temporal distribution of different
246 caliciviruses can help to 1) guide the development, and 2) increase the effectiveness of
247 improved rabbit management programs, while domestic rabbit owners can instigate targeted
248 control and prevention measures when calicivirus is active in their geographical region. We
249 have demonstrated a novel sampling strategy to enable robust and efficient identification of
250 caliciviruses to the virus variant level circulating in Australian lagomorph populations.

bioRxiv preprint doi: https://doi.org/10.1101/585885; this version posted March 25, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

251 There was no significant difference in detection sensitivity between the fly species
252 tested, namely *Calliphora augur*, *C. hilli hilli*, *C. stygia, Chrysomya rufifacies*, *Chrysomya*
253 *varipes* or an unidentified calliphorid species (presumably *C. canimicans bezzii* based on
254 morphological identification). All individuals were obtained from a single collection, and viral
255 RNA was detected in amounts greater than the limit of detection of the assay in all flies
256 tested except a single *Chrysomya rufifacies*. Since it is unlikely that 100% of flies carry rabbit
257 calicivirus at any given point in time, this suggests that contamination between flies occurs
258 within a trap, probably through contact with flyspots excreted from infected flies (Asgari et
259 al., 1998). Detection sensitivity was also found to be independent of the number of individual
260 flies pooled for RNA extraction, at least for pool sizes between one and ten individuals.
261 Presumably, this is due to the virus being concentrated in the digestive tract of flies
262 combined with a dilution effect when additional fly tissue is added. Our choice of pool size
263 now depends on the minimum tissue amount recommended for RNA extraction (20 mg dry
264 fly tissue) and a maximum practical amount for processing (approximately 100 mg of dry fly
265 tissue). Additionally, no significant differences in detection sensitivity were observed when
266 flies were freeze-dried prior to processing, or when alternative buffers were used for tissue
267 homogenisation. Freeze-drying flies facilitates quantification of viral load in flies on a dry
268 matter basis, since flies can vary considerably in their moisture content depending on
269 environmental conditions. Homogenisation is also more thorough for freeze-dried samples,
270 compared to fresh frozen samples. Flies stored in saturated salt solutions (e.g. RNALater)
271 should not be freeze-dried, as the salt crystallises and interferes with quantification and
272 homogenisation.

273 We then used previously described RT-qPCR and strain-specific endpoint RT-PCR
274 assays to monitor rabbit calicivirus circulation in carrion flies over a 22 month period at two
275 locations approximately 35 km apart near Canberra ACT. By testing carrion fly samples in
276 series, initially with the RT-qPCR assay and subsequently with the strain-specific RT-PCR
277 assays for samples >1000 capsid copies per µl of RNA, we were able to identify clear
278 temporal peaks of rabbit calicivirus activity at both sampling locations. Virus variant

bioRxiv preprint doi: https://doi.org/10.1101/585885; this version posted March 25, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

279 identification was hindered by a relatively low positive predictive value of the SYBR-green-
280 based RT-qPCR assay used here, and more sensitive and specific tests may be able to
281 further improve this testing pipeline in the future. Depending on the relative importance of
282 false negative versus false positive results, this threshold for strain identification can be
283 adjusted as required. Testing of multiple replicate pools from a single trap or multiple
284 collections from a specific time point and location may also increase detection sensitivity and
285 specificity, however, this must be balanced against the costs incurred with additional testing.
286 The pattern of positive fly samples was very similar at both Black Mountain and
287 Murrumbateman, with peaks of virus detection in March-April, October, and December-
288 January. This similarity is not surprising, given that the distance between these locations is
289 only 35 km and rabbit caliciviruses can spread at rates of up to 400 km per month during
290 autumn and spring (Kovaliski, 1998). Furthermore, flies are known to move up to 15 km per
291 day (cited in McColl et al., 2002). There was good correlation between peaks of detections in
292 flies and detections in rabbits, at the temporal, geographic, and virus variant levels. Virus
293 was detected in both rabbits and flies in April 2017, October 2017, and January 2018 at
294 Black Mountain and in April and October 2017 at Murrumbateman. Detections in carrion flies
295 typically preceded detection in lagomorphs by around two weeks. The virus detected in flies
296 matched that detected in lagomorphs except during April 2017 at Murrumbateman, where
297 recombinant GI.4e-GI.2 was detected in lagomorph carcasses while GI.2 was detected in
298 flies. All subsequent detections from Murrumbateman were recombinant GI.4e-GI.2.
299 Virus was detected in flies only at Black Mountain in December 2016 and April 2018
300 and Murrumbateman in January and April 2018. This suggests that using carrion flies as
301 proxies to monitor rabbit calicivirus circulation in the environment may in fact be more
302 sensitive than relying on detection of rabbit carcasses. Interestingly, of the two fly samples
303 collected from Black Mountain in December 2016, one was identified as GI.4e-GI.2 while the
304 other was identified as GI.2. Previous detections of calicivirus in rabbit carcasses at Black
305 Mountain were all GI.2, however, recombinant GI.4e-GI.2 was detected at one location in the
306 ACT in a dead rabbit approximately 13 km away in December 2016. It is possible that this

bioRxiv preprint doi: https://doi.org/10.1101/585885; this version posted March 25, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

307 detection at Black Mountain was in fact dispersal of flies from this distant site, or that the two
308 viruses were co-circulating and no rabbit carcass with the recombinant GI.4e-GI.2 was
309 detected. Virus was detected in rabbits only in May 2018 at Black Mountain, which may have
310 been related to the April 2018 detections in flies. Additionally, virus was detected in rabbits
311 only in June 2017 and 2018 in Murrumbateman. Fly samples were challenging to obtain
312 during the winter months, when fly activity is low in this region of Australia. Indeed, no flies
313 were trapped from Murrumbateman during June-July 2017 or May-June 2018. Consideration
314 should be given to an alternative sample source when flies are not active in a given region,
315 for example, over winter.

316 It should be noted that not all fly samples collected during outbreaks were positive.
317 There is a high rate of negative or inconclusive samples, particularly when using the
318 stringent threshold we have selected. This hampers the interpretation of single fly
319 collections. This suggests that sampling of carrion flies to monitor calicivirus activity may be
320 best suited for temporal sampling, with samples collected regularly, and probably not less
321 than once a month, although sampling frequency for optimal monitoring has not been
322 modelled or experimentally determined at this stage. To function as a tool to guide wild
323 management control programs and disease control and prevention programs in domestic
324 rabbits, regular long-term sampling would be required. To instigate a national systematic
325 sampling network, the spatial scales at which calicivirus dynamics change in flies still needs
326 to be investigated. The fly sampling method reported here provides a robust and efficient
327 additional method to monitor broad-scale epidemiological patterns of rabbit calicivirus activity
328 that could be used to help guide wild rabbit management programs.

329

330 **Acknowledgements**

331 The authors wish to thank Roslyn Mourant, Lily Tran, and Gordon Soon for their assistance
332 with sample collection and processing and Adam Croxford for helpful suggestions. We also
333 thank Peter Kerr and Amanda Padovan for critical review of the draft manuscript. This

bioRxiv preprint doi: https://doi.org/10.1101/585885; this version posted March 25, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

334 project was funded by the Commonwealth Scientific and Industrial Research Organisation—
335 Health and Biosecurity.
336
337 **Conflict of Interest**
338 The authors declare no conflicts of interest.
339
340 **References**
341 Asgari, S., Hardy, J. R., Sinclair, R. G., & Cooke, B. D. (1998). Field evidence for mechanical
342 transmission of rabbit haemorrhagic disease virus (RHDV) by flies (*Diptera*:*Calliphoridae*)
343 among wild rabbits in Australia. Virus Research, 54, 123–32. *doi:* 10.1016/S0168-
344 1702(98)00017-3
345 Cooke, B. D., & Fenner, F. (2002). Rabbit haemorrhagic disease and the biological control of
346 wild rabbits, *Oryctolagus cuniculus*, in Australia and New Zealand. Wildlife Research, 29,
347 689–706. *doi*: 10.1071/WR02010
348 Cooke, B. D., Robinson, A. J., Merchant, J. C., Nardin, A., & Capucci, L. (2000). Use of
349 ELISAs in field studies of rabbit haemorrhagic disease (RHD) in Australia. Epidemiology and
350 Infection**,** 124, 563–76. *doi:* 10.1017/S0950268899003994
351 Cox, T. E., Liu, J., van de Ven, R., & Strive, T. (2017). Different serological profiles to co-
352 occurring pathogenic and nonpathogenic caliciviruses in wild European rabbits (*Oryctolagus*
353 *cuniculus*) across Australia. Journal of Wildlife Diseases, 53, 472–481. *doi*: 10.7589/2016-
354 06-148
355 Hall, R.N., J.E. Mahar, A.J. Read, R. Mourant, M. Piper, N. Huang, and T. Strive, 2018: A
356 strain-specific multiplex RT-PCR for Australian rabbit haemorrhagic disease viruses
357 uncovers a new recombinant virus variant in rabbits and hares. *Transbound. Emerg. Dis.* **65**,
358 e444–e456. *doi*: 10.1111/tbed.12779
359 Hall, R. N., Mahar, J. E., Haboury, S., Stevens, V., Holmes, E. C., & Strive, T. (2015).
360 Emerging rabbit hemorrhagic disease virus 2 (RHDVb), Australia. Emerging Infectious
361 Diseases, 21, 2276–2278. *doi*: 10.3201/eid2112.151210

bioRxiv preprint doi: https://doi.org/10.1101/585885; this version posted March 25, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

362 Henning, J., Schnitzler, F. R., Pfeiffer, D. U., & Davies, P. (2005). Influence of weather
363 conditions on fly abundance and its implications for transmission of rabbit haemorrhagic
364 disease virus in the North Island of New Zealand. Medical and Veterinary Entomology, 19,
365 251–62. *doi*: 10.1111/j.1365-2915.2005.00568.x
366 Kovaliski, J. (1998). Monitoring the spread of rabbit hemorrhagic disease virus as a new
367 biological agent for control of wild European rabbits in Australia. Journal of Wildlife Diseases,
368 34, 421–8. *doi*: 10.7589/0090-3558-34.3.421
369 Le Pendu, J., Abrantes, J., Bertagnoli, S., Guitton,, J. S., Le Gall-Reculé, G., Lopes, A. M.,
370 Marchandeau, S., Alda, F., Almeida, T., Célio, A. P., Bárcena, J., Burmakina, G., Blanco, E.,
371 Calvete, C., Cavadini, P., Cooke, B., Dalton, K., Delibes Mateos, M., Deptula, W., Eden, J.
372 S., Wang, F., Ferreira, C. C., Ferreira, P., Foronda, P., Gonçalves, D., Gavier-Widén, D.,
373 Hall, R. N., Hukowska-Szematowicz, B., Kerr, P., Kovaliski, J., Lavazza, A., Mahar, J.,
374 Malogolovkin, A., Marques, R. M., Marques, S., Martin-Alonso, A., Monterroso, P., Moreno,
375 S., Mutze, G., Neimanis, A., Niedzwiedzka-Rystwej, P., Peacock, D., Parra, F., Rocchi, M.,
376 Rouco, C., Ruvoën-Clouet, N., Silva, E., Silvério, D., Strive, T., Thompson, G., Tokarz-
377 Deptula, B., & Esteves, P. (2017). Proposal for a unified classification system and
378 nomenclature of lagoviruses, Journal of General Virology, 98, 1658-66. *doi*:
379 10.1099/jgv.0.000840
380 Mahar, J. E., Read, A. J., Gu, X., Urakova, N., Mourant, R., Piper, M., Haboury, S., Holmes,
381 E. C., Strive, T., & Hall, R.N. (2018). Detection and circulation of a novel rabbit hemorrhagic
382 disease virus in Australia. Emerging Infectious Diseases, 24, 22–31. *doi*:
383 10.3201/eid2401.170412
384 McColl, K. A., Merchant, J. C., Hardy, J., Cooke, B. D., Robinson, A., & Westbury, H.A.
385 (2002). Evidence for insect transmission of rabbit haemorrhagic disease virus. Epidemiology
386 and Infection, 129, 655–63. *doi*: 10.1017/S0950268802007756
387 Invasive Animals CRC. (2014). Import and evaluate new rabbit haemorrhagic disease virus
388 (RHDV) variants to strengthen rabbit biocontrol. Report to the Vertebrate Pests Committee,
389 Canberra, Australia. Retrieved from https://www.pestsmart.org.au/rhd-boost/

bioRxiv preprint doi: https://doi.org/10.1101/585885; this version posted March 25, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

390 Schwensow, N. I., Cooke, B., Kovaliski, J., Sinclair, R., Peacock, D., Fickel, J., & Sommer,
391 S. (2014). Rabbit haemorrhagic disease: virus persistence and adaptation in Australia.
392 Evolutionary Applications, 7, 1056–1067. *doi*: 10.1111/eva.12195
393 Wallman, J. F. (2001). A key to the adults of species of blowflies in southern Australia known
394 or suspected to breed in carrion. Medical and Veterinary Entomology, 15, 433–437. *doi*:
395 10.1046/j.0269-283x.2001.00331.x
396 Wells, J. D., & Sperling, F. A. H. (2001). DNA-based identification of forensically important
397 *Chrysomyinae* (*Diptera*: *Calliphoridae*). Forensic Science International, 120, 110–115. *doi*:
398 10.1016/S0379-0738(01)00414-5

bioRxiv preprint doi: https://doi.org/10.1101/585885; this version posted March 25, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

399 Figure 1: Envirosafe™ fly trap containing commercial bait. To prevent flies contacting the
400 bait the specimen jar was covered by a gauze swab.
401
402 Figure 2: Four to five individual flies of each species (a) or five replicate pools of $n = 3$, 5, or
403 10 flies (b) were tested for the presence of rabbit caliciviruses using RT-qPCR. Statistical
404 comparisons between species were performed using pairwise Wilcoxon tests in the R
405 package “ggpubr” as implemented in R3.5.1. The red dashed line indicates the limit of
406 quantification of the RT-qPCR assay.
407
408 Figure 3: Total RNA was extracted from three replicate pools of ten flies by either the
409 standard extraction method, substituting the homogenisation buffer for 10mM Tris + 10 $\mu l.ml^{-}$
410 ^{1} β-mercaptoethanol or 1x PBS + 10 $\mu l.ml^{-1}$ β-mercaptoethanol, or after freeze-drying pools
411 overnight. Virus load was quantified using RT-qPCR. Statistical comparisons between
412 species were performed using pairwise Wilcoxon tests in the R package “ggpubr” as
413 implemented in R3.5.1. The red dashed line indicates the limit of quantification of the RT-
414 qPCR assay.
415
416 Figure 4: Density plots were used to compare the distributions of positive and inconclusive
417 fly samples as a function of viral load as determined by RT-qPCR. Flies were collected
418 between September 2016 and June 2018 and were screened by a universal lagovirus
419 SYBR-green-based RT-qPCR. Those above the limit of quantification of the assay (i.e. 100
420 capsid copies per µl of RNA) were subtyped using strain-specific endpoint RT-PCR assays.
421 Samples for which strain was unambiguously assigned were classified as positive while
422 those that were negative on endpoint RT-PCR, gave inconsistent results on repeated
423 testing, or only amplified very weak bands were classified as inconclusive.

bioRxiv preprint doi: https://doi.org/10.1101/585885; this version posted March 25, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

424

425 Figure 5: Flies were collected regularly from two locations around the ACT between
426 September 2016 and June 2018 to monitor rabbit calicivirus circulation in the environment.
427 Total RNA was extracted and viral load was quantified by RT-qPCR. For samples with viral
428 loads >1000 capsid copies per µl of RNA, virus variant was determined by strain-specific
429 endpoint RT-PCR. Lagomorphs (i.e. rabbits or hares) found dead at these locations over the
430 sampling period and that were positive for rabbit calicivirus were plotted as points at 1×10^6
431 capsid copies per µl of RNA for visualisation purposes.

a)

Log RT-qPCR titre, capsid copies

5
4
3
2
1

ns ns ns ns ns ns

C (unidentified)
C augur
C hilli hilli
C stygia
Ch ruffifacies
Ch varipes

Species

b)

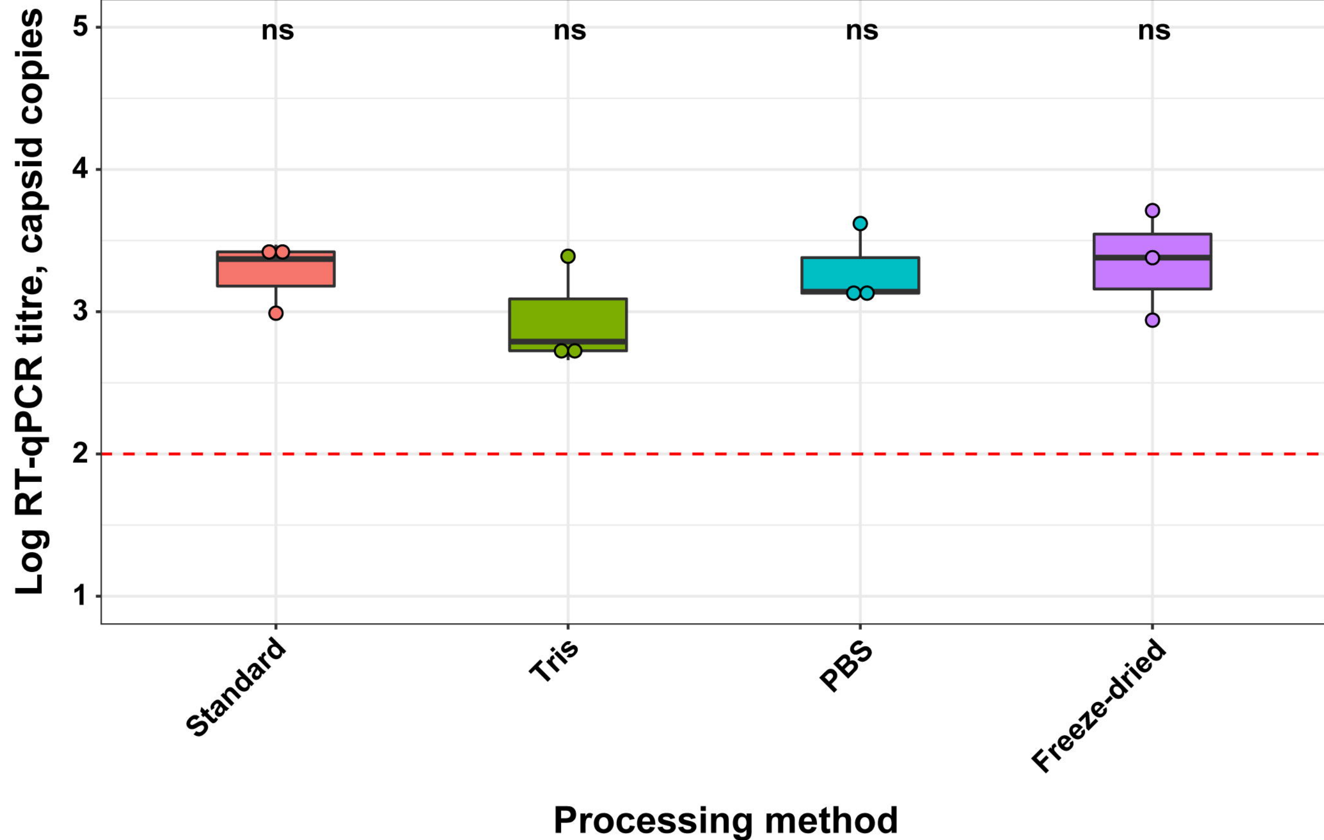
Log RT-qPCR titre, capsid copies

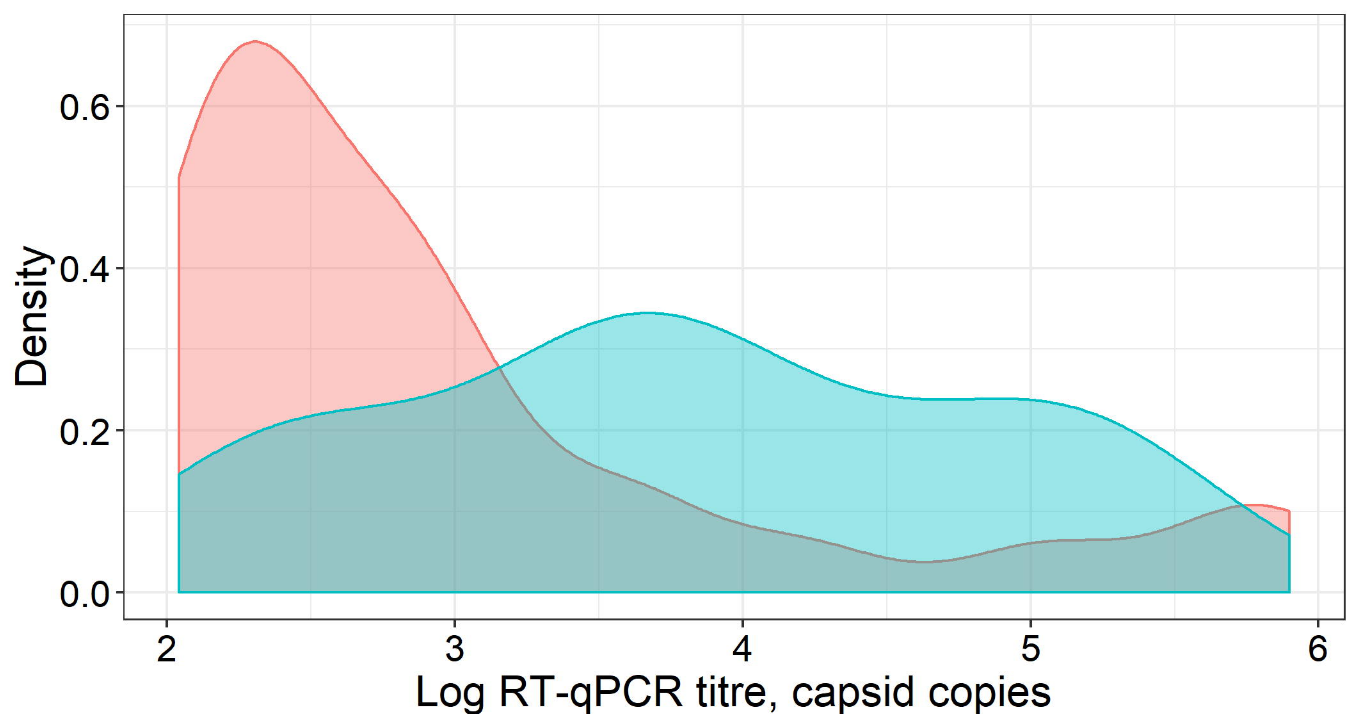
5
4
3
2
1

ns ns ns

Three
Five
Ten

Pool size, number of individual flies


Log RT-qPCR titre, capsid copies
5
4
3
2
1
ns
ns
ns
ns
Standard
Tris
PBS
Freeze-dried
Processing method


Density
0.0
0.2
0.4
0.6
2
3
4
5
6
Log RT-qPCR titre, capsid copies
Endpoint PCR result:
Inconclusive
Positive

Black Mountain

Murrumbateman

Log RT-qPCR titre, capsid copies

0
2
4
6

Sep-2016
Oct-2016
Nov-2016
Dec-2016
Jan-2017
Feb-2017
Mar-2017
Apr-2017
May-2017
Jun-2017
Jul-2017
Aug-2017
Sep-2017
Oct-2017
Nov-2017
Dec-2017
Jan-2018
Feb-2018
Mar-2018
Apr-2018
May-2018
Jun-2018
Jul-2018

Collection date

Endpoint RT-PCR, rabbits/hares:

GI.2

GI.4e-GI.2

Endpoint RT-PCR, flies:

GI.2

GI.4e-GI.2

Inconclusive

N/A