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1 **Adenovirus: An emerging factor in red squirrel *Sciurus vulgaris***
2 **conservation**

3

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21

22 **ABSTRACT**

23

24 1. Adenovirus is an emerging threat to red squirrel *Sciurus vulgaris*
25 conservation, but confirming clinically-significant adenovirus infections in
26 red squirrels is challenging. Rapid intestinal autolysis after death in wild
27 animals frequently obscures pathology characteristic of the disease in
28 animals found dead.

29 2. We review the available literature to determine current understanding of
30 both sub-clinical and clinically significant adenovirus infections in free-
31 living wild and captive red squirrel populations.

32 3. Benefits of scientific testing for adenovirus incorporating both
33 transmission electron microscopy (TEM) and polymerase chain reaction
34 (PCR) technologies are compared and contrasted. We favour viral
35 particle detection using TEM in animals exhibiting enteropathy at post
36 mortem and the use of PCR to detect sub-clinical cases where no
37 enteric abnormalities are observed.

38 4. Adenoviral infections associated with re-introduction studies are
39 evaluated by examination of sporadic cases in wild populations and of
40 data from captive collections used to service such studies.

41 5. The paucity of data available on adenovirus infection in grey squirrel
42 *Sciurus carolinensis* populations is documented and we highlight that
43 although sub-clinical virus presence is recorded in several locations in

44 Britain and Italy, no clinically-significant disease cases have been
45 detected in the species thus far.

46 6. Current speculation for potential inter-specific infection between sciurids
47 and other woodland rodents such as wood mice *Apodemus sylvaticus* is
48 examined. Where sub-clinical adenovirus presence has been detected
49 in sympatric populations occupying the same point food sources,
50 husbandry methods may be used to diminish the potential for cross-
51 infection.

52 7. Our findings highlight the importance of controlling disease in red
53 squirrel populations by using clearly defined scientific methods. In
54 addition, we propose hypothetical conservation benefits of restricting
55 contact rates between red squirrels and sympatric grey squirrels and of
56 limiting competition from other woodland rodent species.

57

58 **KEY WORDS: adenovirus infection, conservation, disease, grey squirrel,**
59 **red squirrel**

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68 **INTRODUCTION**

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70 Historically, disease was not recognized as a mechanism by which red squirrels

71 *Sciurus vulgaris* were replaced by grey squirrels *S. carolinensis* in a landscape.

72 Indeed, it was unclear initially whether the larger grey squirrel was directly

73 involved at all, was taking advantage of space vacated by natural fluctuations in

74 red squirrel population, or ultimately was better adapted to a larger range of

75 habitats (Middleton, 1931). Disease enzootics that were recorded in red squirrel

76 populations were notable for encompassing areas where grey squirrels were

77 absent (Shorten, 1954). Gurnell, (1987) noted “no evidence that grey squirrels

78 brought with them a disease which is causing the downfall of the red”.

79

80 By the 1990s, research had focussed heavily upon resource competition (Gurnell

81 and Pepper, 1993), including inter-specific differences in the relative efficiency

82 with which some tree seed is digested (Kenward and Holm, 1993). Inter-specific

83 resource competition, (Wauters *et al.* 2005), negative impacts on red squirrel

84 juvenile recruitment rates (Gurnell *et al.* 2004) and the effects of seed cache

85 piracy (Wauters *et al.* 2002) are today recognised as major contributors to red

86 squirrel extinction in sympatric populations.

87

88 However, progressive advances in viral research subsequently established that

89 grey squirrels carry the Squirrel pox virus (SQPV) as a sub-clinical infection, and

90 that inter-specific infection in sympatric red squirrels leads to epizootic disease
91 which is a significant factor in regional population declines in the UK (Rushton *et al.*
92 *et al.* 2006; Sainsbury *et al.* 2008; Carroll *et al.* 2009; Bruemmer *et al.* 2010). Even
93 more recently, adenovirus infection has been identified increasingly as a cause of
94 mortality in free-living wild and captive red squirrel populations. An expanding
95 geographic distribution of cases has been revealed, affecting not only wild
96 populations, but increasingly being associated with high mortalities in captive
97 collections used as both breeding stock and for use in wild population re-
98 enforcement programmes. Additionally, grey squirrels have now been identified
99 as a sub-clinical carrier of the adenovirus among sympatric populations (Everest
100 *et al.* 2009; Romeo *et al.* 2014).

101

102 Blood analyses, using enzyme linked immuno sorbent assay (ELISA) and tissue
103 polymerase chain reaction (PCR) techniques, are routinely applied to determine
104 SQPV infection in both squirrel species. Parallel transmission electron
105 microscopy (TEM) screening of skin lesion material can be used to confirm the
106 presence of pox viral particles in typical advanced red squirrel cases. However, in
107 contrast to the detection of SQPV, the detection of adenovirus-associated or
108 clinically-significant adenovirus cases among red squirrels is challenging, due to
109 an absence or non-specificity of external clinical signs of disease. Until relatively
110 recently little was known about this infection in either squirrel species, or its
111 significance in red squirrel declines. Due to increasing scientific activity, both as a
112 retrospective exercise and proactive surveillance, a wider picture is gradually

113 emerging of the evolving impact that this virus is having with respect to both
114 sporadic disease cases in free-living wild squirrels across Great Britain and in red
115 squirrel re-introduction and captive breeding programmes. However, the mere
116 presence of adenovirus in the body does not signify disease. The virus may
117 indeed be present as a clinically significant infection, causing the death of the
118 animal; in this case viral particles can be detected by TEM in faecal material or
119 viral DNA can be amplified from tissue material such as the spleen. Adenovirus
120 can also be present as an asymptomatic infection or transience presence,
121 causing no apparent disease signs or indications of ill health, and the animal may
122 be outwardly healthy. Subsequent death due to an unrelated problem could then
123 show the presence of the amplified viral DNA by PCR analysis, whereas TEM
124 would fail to detect any viral particles.

125

126 Adenovirus infection damages the villi in the red squirrel intestinal mucosa, but
127 autolysis within hours of death typically confounds histological examination, by
128 precluding detection of characteristic adenovirus inclusion bodies (Erdélyi and
129 Duff, 2012), as seen in Fig. 1. By TEM on ultra-thin sections, these inclusions
130 have been shown to contain abundant viral particles (Fig. 2, arrowed). The
131 findings of enteropathy or diarrhoea are non-specific and are associated with
132 several other diseases (Everest *et al.* 2010a). While it is difficult to obtain
133 histologically-adequate gut wall samples prior to autolysis, experience shows that
134 gross pathological changes indicative of enteropathy, such as liquid intestinal
135 content, correlate strongly with gut viral particle detection by TEM (Fig. 3). The

136 presence of viral particles is therefore considered strongly suggestive of
137 clinically-significant infection (Everest *et al.* 2012b). Nonetheless, in the most
138 autolysed wild squirrel cases, pathologists may assume intestinal material to be
139 of such limited value that it is not retained, even though archival samples of other
140 tissues such as liver or spleen may be. Our understanding of the temporal and
141 spatial scope of clinical adenovirus infection (Fig. 4) has recently been improved
142 through more frequent proactive and reactive post mortem screening, in
143 particular with TEM application.

144

145 We review the current understanding of infection and disease in red squirrels,
146 grey squirrels and other small rodents such as wood mice *Apodemus sylvaticus*,
147 with particular reference to the UK, and highlight key areas for future adenovirus
148 infection research that have particular relevance to the applied conservation of
149 the red squirrel.

150

151 **RED SQUIRRELS**

152

153 **The geographical distribution of adenovirus infection**

154

155 The first reports in the literature of adenovirus in free-living wild red squirrels from
156 Great Britain were recorded from Suffolk (Sainsbury *et al.* 2001) and Cumbria,
157 England (Duff *et al.* 2007), then from Wales (Everest *et al.* 2008), Scotland
158 (Everest *et al.* 2010a) and finally from Northern Ireland (Everest *et al.* 2012a),

159 demonstrating a wide geographical distribution (Fig. 4; Table 1). Retrospective
160 national surveillance of red squirrel mortalities across Great Britain, reported by
161 Martínez-Jiménez *et al.*, (2011) revealed that 60 (12%) of 493 cases showed
162 enteric signs. Of these 60, 13 animals, all of which were exhibiting diarrhoea,
163 were selected for analysis by TEM. Of these 13, two animals (15%; Table 1), one
164 from Cumbria, the other from Lancashire, England were confirmed as adenovirus
165 cases by the TEM detection of viral particles. In another retrospective study,
166 adenovirus particles were identified by TEM in 10 (14%) of 70 free-living wild red
167 squirrels where enteropathy was suspected, from Cumbria, Lancashire and
168 Northumberland, England and Anglesey, Wales (Everest *et al.* 2010b; Table 1).
169 However, given the opportunistic sampling of post mortem cases and the paucity
170 of data from living animals, it is difficult to interpret the importance of adenovirus
171 as an overall contributor to mortality from these studies alone.

172

173 Sainsbury *et al.*, (2001) and Martínez-Jiménez *et al.*, (2011) both reported on a
174 population re-enforcement study at Thetford Chase (Suffolk, England) in the late
175 1990s, with animals that had been trans-located from Cumbria and had
176 contracted the infection and died in 1997 (Table 1). These animals may have
177 been under stress that could have influenced the course of the disease.

178 Diarrhoea was associated with each of 10 adenovirus cases recorded in red
179 squirrels and intestinal haemorrhage or inflammation was observed in seven
180 cases. The extant Thetford Chase wild red squirrel population at that time was
181 judged to consist of 10 to 20 individuals (no more than 40, Gurnell *et al.* 1997),

182 and consequently adenovirus infection was a notable factor in the study.
183 Subsequent research, (Everest *et al.* 2012b; Table 1) has revealed adenovirus
184 infection to be associated with a high proportion of deaths in squirrels housed in
185 captive collections in Wales, indicating that viral epizootics can be locally
186 significant.

187

188 Of 13 captive deaths sampled from the re-introductions on the island of
189 Anglesey, situated off the North Wales coast, 12 (92%) were confirmed as
190 positive for the virus (three detecting viral particles by TEM and nine amplifying
191 viral DNA by PCR). Samples from 16 captive deaths at the Welsh Mountain Zoo,
192 Colwyn Bay, Wales (TWMZ) revealed viral DNA amplified by PCR in 14 (88%)
193 cases (Everest *et al.* 2012b). In a further 24 captive deaths originating from
194 zoological collections in England, for which tissue, faecal or intestinal content
195 samples were available, 20 (83%), were observed to be positive for adenovirus
196 (Everest *et al.*, unpublished; Table1).

197

198 Analyses performed on 31 free-living wild red squirrels found dead on Anglesey
199 revealed that 13 (42%) were positive for the virus. Of these positive cases, seven
200 (54%), originated from within Newborough Forest and from these, five (71%),
201 were identified as positive by PCR analyses, three of which also tested negative
202 by TEM. One (14%), contained viral particles when analysed by TEM only, and
203 one case was confirmed by both tests. The remaining six cases were from other
204 Anglesey coniferous and broad-leaved woodlands; all were detected as viral

205 DNA carriers by PCR, but negative for viral particles by TEM (Everest *et al.*
206 2012b).

207

208 In the latest published report of adenovirus in red squirrels from Great Britain,
209 Everest *et al.* (2013) record that nine (45%) of 20 animals were identified as
210 positive for the virus through amplification of viral DNA by PCR. These animals
211 derived from locations on the Isle of Wight, Jersey and Brownsea Island, all
212 islands off Great Britain without grey squirrels. Intestinal content samples from 12
213 of these 20 animals were originally examined by TEM and found to be negative
214 for virus particles (Everest *et al.* 2010b). This shows the benefit of using parallel
215 TEM and PCR screening to determine sub-clinical virus presence, which can
216 easily go undetected.

217

218 There are very limited reports of adenovirus outbreaks in red squirrels from
219 outside the UK. One, involving three deaths, was from a captive collection in
220 Germany (Peters *et al.* 2011); in the other, 77 road traffic accident carcasses
221 from Italy were examined by a combination of TEM and PCR analyses (Romeo *et*
222 *al.* 2014). Twelve (16%) were positive for amplified viral DNA by PCR (Table 1).
223 As with the outbreak in Germany (Peters *et al.* 2011), and unlike the situation in
224 most of Great Britain, viral presence was detected in red squirrel populations
225 from areas where the grey squirrel was not known to be present in the immediate
226 landscape.

227

228 **Adenovirus presence determined in deaths by other causes**

229

230 Traumatic deaths, such as drowning and road traffic accidents, have revealed
231 animals positive for amplification of viral DNA by PCR, but negative for faecal
232 viral particle detection by TEM. These cases occur in animals which lack enteric
233 abnormalities at post mortem examination. These findings suggest that sub-
234 clinical infections are present and may be widespread within wild British
235 populations of red squirrels (Everest *et al.* 2012b; Table 1).

236

237 **Adenovirus strain speciation**

238

239 Phylogenetic analysis demonstrates that adenovirus sequences from squirrel
240 samples cluster with mastadenoviruses but are distinct from other adenoviruses
241 within the genus (Sainsbury *et al.* 2001, Peters *et al.* 2011), although squirrel
242 adenovirus has not yet been approved as a species (King *et al.* 2011).
243 Sequencing has revealed a lack of adenovirus strain variability. The identity of
244 the adenovirus in a partial fragment of the hexon gene from the German outbreak
245 (GU735084) described by Peters *et al.*, (2011) was identical to the putative
246 Suffolk strain (Sainsbury *et al.* 2001). In contrast, in those cases described by
247 Everest *et al.* (2012b), sequences were detected which were identical to those
248 found in Cumbrian cases from 2007 (JN205244.1). Everest *et al.* (2012b) used a
249 partial fragment of the polymerase gene, which in turn identified cases that were
250 genetically identical to the grey squirrel cases detected on Anglesey (Everest *et*

251 *al.* 2009). This is remarkable, as the cases were separated both spatially and
252 temporally. It has been suggested, therefore, that the viruses involved in each of
253 these cases are very closely related, or perhaps identical (Peters *et al.* 2011).

254

255 In general, the samples described above have not been randomly sourced and
256 case selection was influenced by carcass suitability and value in terms of post
257 mortem examination. This is particularly true for captive collections, where the
258 prevailing close confinement within enclosures would have allowed for easy
259 spread of the virus between individual animals, thus accounting for the
260 apparently high incidence of infection in such collections.

261

262

263 **GREY SQUIRRELS**

264

265 Given the role that grey squirrels play in SQPV infection in red squirrel
266 populations, it is natural to investigate whether sympatric grey squirrel
267 populations are also a source of inter-specific adenovirus infection.

268 Romeo *et al.*, (2014) found PCR amplified adenoviral DNA in only two (1%) of
269 232 grey squirrels from Italy. Screening of tissues from wild adult grey squirrels
270 (n=18) trapped and euthanased at the Welsh Mountain Zoo in 2011 failed to
271 reveal viral particles in the gut by TEM (which would have suggested clinically-
272 significant infection), yet 10 of these 18 animals (56%) were positive by PCR
273 analyses on spleen tissue, (Everest *et al.* unpublished) and were hence

274 determined as adenovirus carriers. Although the numbers of animals were small
275 in this study, the PCR figure is very similar to the 60% sero-prevalence reported
276 by Greenwood and Sanchez, (2002) using murine adenovirus ELISA tests for
277 antibodies in grey squirrels from the same zoo; a location where dead captive red
278 squirrels have been found with enteric symptoms and viral particles in the
279 intestinal tract.

280

281 At the Newborough Forest re-introduction site on Anglesey, adenovirus DNA was
282 detected by PCR analysis from two grey squirrels caught in 2006 (Everest *et al.*
283 2009). Wider PCR screening of archived and proactively-sourced blood
284 sampling, involving over 200 samples and thus forming a study larger than that
285 reported by Romeo *et al.* (2014), was subsequently undertaken and reported by
286 Everest *et al.* (2012b, Table 2) for both Anglesey locations and woodland in
287 Gwynedd, North Wales, within a few kilometres of the Menai Straits. Spleen
288 tissue collected from the Gwynedd site was examined in 2012 (Everest *et al.*
289 unpublished), and amplification of DNA revealed a much higher percentage of
290 positives (54%) than in blood (7%, see Table 2).

291

292 The 2012 Gwynedd result (Everest *et al.* unpublished) was further confirmed,
293 when both spleen and blood were available for analysis from each of 14 adult
294 grey squirrels trapped at the Welsh mountain Zoo. Adenovirus DNA was detected
295 from spleen tissue in eight cases (57%), but there were no positive results from
296 the 14 blood samples from the same animals (Everest *et al.* unpublished), thus

297 demonstrating that source tissue type is an important consideration in adenovirus
298 screening.

299

300 Historically, assessing infection in grey squirrels is challenging, as previously
301 reported blood based testing was serologically based. Thus exposure to the virus
302 could result in potentially long-lasting sero-conversion, although this may wane
303 with age. In contrast, an animal may be viraemic (and therefore PCR-positive) for
304 only a short period, meaning PCR analyses have only a small time window to be
305 effective for viral diagnosis. In this context, serologically- based ELISA analyses
306 may be more sensitive in nature than PCR techniques.

307

308 Although evidence of infection has been found, no clinically-significant cases of
309 adenovirus have been identified to date in grey squirrels and viral particles have
310 been absent from intestinal content examined by TEM studies of grey squirrels
311 from Cumbria ($n=36$), Wales ($n=58$, Everest *et al.* unpublished), Thetford Chase
312 study ($n=10$, Martínez-Jiménez *et al.* 2011) and Italy ($n=3$, Romeo *et al.* 2014).

313

314

315 **SMALL RODENTS**

316

317 Peters *et al.*, (2011), documented adenovirus infection by TEM in a captive red
318 squirrel collection from Germany, and red squirrel infections have been recorded
319 on both the Isle of Wight and Jersey (Everest *et al.* 2013), all of which are regions

320 where the grey squirrel is absent. Additionally, Romeo *et al.*, (2014) documented
321 infections in red squirrels in areas where the grey squirrel was not known to be
322 present. This means that alongside intra-specific and potential grey squirrel to
323 red squirrel infections, inter-specific infection from other small rodents such as
324 wood mice is possible.

325

326 In order to investigate this potential infection route, Everest *et al.*, (2013)
327 examined the spleens of wood mice trapped on the Island of Anglesey for the
328 presence of adenovirus by PCR analyses. Adenoviral DNA was amplified from
329 three of 15 mice (20%), trapped at two woodland sites which had red squirrel
330 feeding stations and where cases of clinically-significant adenovirus infection of
331 wild red squirrels had been recorded. Two of 24 (8%) mice trapped in north
332 Wales within woodland enclosures housing captive red squirrels also tested
333 positive for adenovirus by PCR. Our results therefore demonstrate the potential
334 for adenovirus infection in sympatric communities of grey squirrel, red squirrel
335 and wood mice.

336

337 The PCR primers used to test the wood mice samples had been designed based
338 on a sequence of the adenoviral DNA polymerase gene from squirrel samples
339 (JN205244.1; Everest *et al.* 2012). However, further investigations into whether
340 these primers would detect other adenoviruses were not undertaken.

341 It is therefore unclear at present whether the strain detected in mice is identical to
342 that detected in squirrels, and so further molecular sequencing is required.

343 Greenwood and Sanchez (2002) used an ELISA derived for the serological
344 detection of murine adenoviruses to detect adenovirus in grey squirrels.
345 Therefore, it is possible that either cross reactivity exists between species-
346 specific viruses from the two species, or an identical virus infects both.

347

348

349 **DISCUSSION**

350

351 Retrospective study of archived tissue and blood samples (Everest *et al.* 2010;
352 2012b; Martínez-Jiménez *et al.* 2011) has advanced our understanding of both
353 the temporal and spatial distribution of adenovirus infection within red squirrel
354 populations. Recent examination of trauma deaths has also revealed sub-clinical
355 infections in wild individuals at the time of death, namely negative TEM results for
356 adenovirus particles in faecal and intestinal samples but positive results for viral
357 DNA from tissues by PCR analysis (Everest *et al.* 2012b; Romeo *et al.* 2014).
358 Much however, remains unclear about the epizootiology, in particular, the roles of
359 squirrel population density and stress. Currently, much of our understanding is
360 based upon captive collections and there is therefore also a pressing research
361 need to investigate the distribution and effects of the infection among wild red
362 and grey squirrel populations. Opportunities for application of a qPCR technique
363 to quantitate virus load in faeces, tissues and blood in order to partition
364 pathological from asymptomatic infections would also be beneficial.

365

366 Research in North Wales (Everest *et al.* 2009; 2012b; Greenwood and Sanchez,
367 2002), and in Italy by Romeo *et al.*, (2014) has demonstrated adenovirus
368 infection or exposure in grey squirrels, but whether this has any clinical
369 significance in these populations remains unknown. To this end, a controlled
370 challenge experiment in grey and red squirrels using the same virus isolate would
371 also help to advance our understanding.

372

373 There is also a paucity of data on adenovirus prevalence within regional grey
374 squirrel populations in the UK. An annual survey combining PCR and TEM
375 analyses was limited solely to squirrel populations in North Wales. Additional
376 regional studies of this type would therefore be useful

377

378 Given that grey squirrels appear to be infected with both adenovirus and SQPV,
379 the accepted management practice of removing grey squirrel populations to
380 control SQPV infections would also mitigate the potential for adenovirus infection.
381 Additionally, conservation managers could potentially evolve protocols to combat
382 potential infection pathways involving other woodland rodents such as wood
383 mice, although there may be a significant cost implication to this approach. On
384 Anglesey, adenovirus infection risk was highlighted as a major difficulty faced
385 during the re-introduction of red squirrels to Newborough Forest (Shuttleworth *et*
386 *al.* 2008). Release protocols have been modified with animals now housed for
387 only a few weeks, during which faecal and blood screening is undertaken for
388 adenovirus (Shuttleworth, 2010). More widely, it has been recommended that

389 hygiene protocols at supplemental feeding hoppers routinely focus upon limiting
390 adenovirus infection via faecal-oral routes (Everest *et al.* 2012b). Given our
391 recent findings and because of the potential for transmission of other rodent-
392 borne infections, this should encompass mouse control.

393

394 If wood mice act as an infection reservoir, there are obvious implications for
395 scenarios that concentrate their activities at point food sources such as garden
396 bird tables or supplemental feed hoppers also visited by red squirrels. It may
397 therefore be prudent to place red squirrel supplemental feed hoppers on posts
398 with cone shaped baffles near the base to prevent mice from accessing the
399 hopper above, instead of, as is common practice, fixing hoppers to tree trunks,
400 which allows mice easy access. Accumulation of discarded shells and food
401 remains beneath hoppers should be minimised. Trapping protocols should
402 include regular disinfection of all traps, not only those that have contained grey
403 squirrels, so as to limit any potential mouse to red squirrel inter-specific virus
404 transmission.

405

406

407 **CONCLUSIONS**

408

409 This review of the available evidence within the published literature, coupled with
410 recent findings, lead us to conclude that adenovirus should be regarded as a
411 serious disease threat to the various red squirrel re-introduction and captive

412 breeding programmes, and to red squirrel populations in places where grey
413 squirrels, red squirrels and wood mice can interact at point food sources. We also
414 conclude that TEM, while excellent at detecting clinically-significant infection from
415 intestinal samples, is not as sensitive as PCR for detecting sub-clinical
416 adenovirus cases, and that spleen tissue is a better material to screen by PCR
417 than blood. ELISA-based assay on blood samples is the only test available for
418 live animals at present. To address practical and potentially also welfare
419 considerations, alternative assay platforms should be investigated for live animal
420 testing. We would also recommend that disease investigations and adenoviral
421 infection surveillance extend to all three rodent species identified in this review,
422 and where possible, include parallel PCR and TEM sample testing of tissue and
423 intestinal content samples, respectively.

424

425

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427

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440

441

442 **REFERENCES**

443

444 Bruemmer CM, Rushton SP, Gurnell J, Lurz PWW, Nettleton P, Sainsbury AW,
445 Duff JP, Gilray J, McInnes CJ (2010) Epidemiology of squirrel pox virus in grey
446 squirrels in the UK. *Epidemiology and Infection* **138**: 941-950

447

448 Carroll B, Russell P, Gurnell J, Nettleton P, Sainsbury AW (2009) Epidemics of
449 squirrel pox virus disease in red squirrels (*Sciurus vulgaris*): temporal and
450 serological findings. *Epidemiology and Infection* **137**: 257-265

451

452 Duff J, Higgins R, Farrelly S (2007) Enteric adenovirus infection in a red squirrel
453 (*Sciurus vulgaris*). *Veterinary Record* **160**: 384

454

455 Erdélyi K, Duff JP. Adenovirus infection in squirrels. In: Gavier-Widen D, Duff JP
456 and Meredith A (eds) *Infectious diseases of wild animals and birds in Europe*
457 Wiley, UK

458

459 Everest DJ, Butler H, Blackett T, Simpson VR, Shuttleworth CM (2013)

460 Adenovirus infection in red squirrels in areas free from grey squirrels. *Veterinary*

461 *Record* **173**: 199-200

462

463 Everest DJ, Grierson SS, Meredith AL, Milne EM (2010a) Adenovirus in a red

464 squirrel (*Sciurus vulgaris*) from Scotland. *Veterinary Record* **167**: 184

465

466 Everest DJ, Grierson SS, Stidworthy MF, Shuttleworth C (2009) PCR detection of

467 adenovirus in grey squirrels on Anglesey. *Veterinary Record* **165**: 482

468

469 Everest DJ, Griffin J, Warnock ND, Collins L, Dick J, Reid N, Scantlebury M,

470 Marks N, Montgomery I (2012a) Adenovirus particles from a wild red squirrel

471 (*Sciurus vulgaris*) from Northern Ireland. *Veterinary Record* **170**:188

472

473 Everest DJ, Shuttleworth CM, Grierson SS, Duff JP, Jackson N, Litherland P,

474 Kenward RE, Stidworthy MF (2012b) A systematic assessment of the impact of

475 adenovirus infection on a captive re-introduction project for red squirrels (*Sciurus*

476 *vulgaris*). *Veterinary Record* **171**: (7)176

477

478 Everest DJ, Stidworthy MF, Milne EM, Meredith AL, Chantrey J, Shuttleworth

479 CM, Blackett T, Butler H, Wilkinson M, Sainsbury AW (2010b) Retrospective

480 detection by negative contrast electron microscopy of faecal viral particles in wild

481 red squirrels (*Sciurus vulgaris*) with suspected enteropathy in Great Britain.
482 *Veterinary Record* **167**: 1007-1010
483
484 Everest DJ, Stidworthy MF, Shuttleworth C (2008) Adenovirus-associated
485 mortalities in red squirrels (*Sciurus vulgaris*) on Anglesey. *Veterinary Record*
486 **163**: 430
487
488 Greenwood AG and Sanchez S (2002) Serological evidence of murine pathogens
489 in wild grey squirrels (*Sciurus carolinensis*) in north Wales. *Veterinary Record*
490 **150**: 543-546
491
492 Gurnell J. (1987) *The natural history of Squirrels*. p 162 Christopher Helm,
493 London.UK
494
495 Gurnell J. and Pepper H. (1993). "A critical look at conserving the British Red
496 Squirrel (*Sciurus vulgaris*)."
497 *Mammal Review* **23**: (3-4): 127-137
498
499 Gurnell J, Sainsbury AW, Venning T. (1997) Conserving the red squirrel in
500 Thetford Forest. p 59 English Nature Research Report, Peterborough, UK
501
502 Gurnell J, Wauters L, Lurz PWW, Tosi G (2004) Alien species and inter-specific
503 competition: effects of introduced eastern grey squirrels on red squirrel
population dynamics. *Journal of Animal Ecology* **73**: 26-35

504

505 Kenward RE. and Holm JL. (1993). On the replacement of the Red Squirrel in
506 Britain: A phytotoxic explanation. Proceedings of the Royal Society Series B
507 **251**: 187-194

508

509 King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ (eds; 2011) *Virus*
510 *Taxonomy: Classification and Nomenclature of Viruses*. Ninth Report of the
511 International Committee on Taxonomy of Viruses. Elsevier, Amsterdam, The
512 Netherlands.

513

514 Martínez-Jiménez D, Graham D, Couper D, Benkö M, Schöniger S, Gurnell J,
515 Sainsbury AW (2011) Epizootiology and pathologic findings associated with a
516 newly described adenovirus in the red squirrel, (*Sciurus vulgaris*). *Journal of*
517 *Wildlife Diseases* **47**: 442-454

518

519 Middleton AD. (1931) *The Grey Squirrel*. p78-80 Sidgwick and Jackson Ltd,
520 London. UK

521

522 Peters M, Vidovszky MZ, Harrach B, Fischer S, Wohlsein P, Kilwinski J (2011)
523 Squirrel adenovirus type 1 in red squirrels (*Sciurus vulgaris*) in Germany.
524 *Veterinary Record* **169**: 182

525

526 Romeo C, Ferrari N, Rossi C, Everest DJ, Grierson SS, Lanfranchi P, Martinoli A,
527 Saino N, Wauters LA, Hauffe HC. (2014) Ljungan virus and an adenovirus in
528 Italian squirrel populations. *Journal of Wildlife Diseases* **50**: (2) in press, DOI:
529 10.7589/2013-10-260
530
531 Rushton SP, Lurz PWW, Gurnell J, Nettleton P, Bruemmer C, Shirley MDF,
532 Sainsbury AW (2006) Disease threats posed by alien species: the role of a
533 poxvirus in the decline of the native red squirrel in Britain. *Epidemiology and*
534 *Infection* **134**: 521-533
535
536 Sainsbury AW, Adair B, Graham D, Gurnell J, Cunningham AA, Benko M, Papp
537 T (2001) Isolation of a novel adenovirus associated with splenitis, diarrhoea and
538 mortality in trans-located red squirrels, (*Sciurus vulgaris*). *Verhandlungs Bericht*
539 *über die Erkrankung der Zootiere* **40**: 265-270
540
541 Sainsbury AW, Deaville R, Lawson B, Cooley WA, Farrelly SS, Stack MJ, *et al.*
542 (2008) Pox viral disease in red squirrels (*Sciurus vulgaris*) in the UK: spatial and
543 temporal trends of an emerging threat. *Ecohealth* **5**: 305-316
544
545 Shorten MR. (1954) *Squirrels*. p 71 Collins, London UK
546
547 Shuttleworth CM (2010) Turning the grey tide: progress in red squirrel recovery.
548 *Ecos* **31**: 27-35

549

550 Shuttleworth CM, Kenward RE, Jackson N (2008) Re-introduction of the red
551 squirrel into Newborough forest on the island of Anglesey, UK. In: Soorae PS
552 (ed) Global re-introduction perspectives: Re-introduction case-studies from
553 around the globe. 163-166 IUCN/SSC Re-introduction Specialist Group, Abu
554 Dhabi UAE

555

556 Wauters LA, Tosi G, Gurnell J, (2002) Inter-specific competition in tree squirrels:
557 do introduced grey squirrels (*Sciurus carolinensis*) deplete tree seeds hoarded by
558 red squirrels (*Sciurus vulgaris*)? *Behavioral Ecology and Sociobiology* **51**: 360-
559 367

560

561 Wauters LA, Tosi G, Gurnell J, (2005) A review of the competitive effects of alien
562 grey squirrels on behavior, activity and habitat use of red squirrels in mixed
563 deciduous woodland in Italy: *Hystrix Italian Journal of Mammalogy* (n.s.) **16**: (1)
564 27-40

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572 **Table 1.** Test results for adenovirus from tissues or intestinal or faecal content
 573 material from red squirrels *Sciurus vulgaris*.

574

Reference	Study location	Number Tested	Number (%) Positive
Duff <i>et al.</i> 2007	Cumbria wild	2	2/2(100%)
Everest <i>et al.</i> 2008	Anglesey captive	3	3/3 (100%)
Everest <i>et al.</i> 2010a	Scotland wild	1	1 (100%)
Everest <i>et al.</i> 2012a	Northern Ireland wild	2	1/2 (50%)
Martínez-Jiménez <i>et al.</i> 2011	Great Britain wild	13	2/13 (15%)
Martínez-Jiménez <i>et al.</i> 2011	Suffolk captive	10	10/10 (100%)
Everest <i>et al.</i> 2010b	Great Britain wild	70	10/70 (14%)
Sainsbury <i>et al.</i> 2001	Suffolk captive	6	3/6 (50%)
Everest <i>et al.</i> 2012b	Anglesey captive	13	12/13 (92%)
Everest <i>et al.</i> 2012b	Zoo captive Wales	16	14/16 (88%)
Everest <i>et al.</i> 2012b	Anglesey wild	31	13/31 (42%)
Everest, <i>et al.</i> unpublished	England captive	24	20/24 (83%)
Everest <i>et al.</i> 2013	Isle of Wight/ Jersey wild	20	9/20 (45%)
Peters <i>et al.</i> 2011	Germany captive	3	3/3 (100%)
Romeo <i>et al.</i> 2014	Italy wild	77	12/77 (16%)

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578 **Table 2.** Positive PCR test results for adenovirus DNA from blood and spleen
579 tissue from grey squirrels *Sciurus carolinensis* in North Wales. Total number
580 tested and percentage positive are shown.

581

	Anglesey		Gwynedd (Bangor Area)	
	Blood	Spleen	Blood	Spleen
2007 ¹	0% (55)	-	-	-
2010 ¹	23% (26)	-	21% (39)	-
2011 ²	-	-	10% (48) ³	-
2012 ²	25% (4)	-	7% (15)	54% (35)

582

583 ¹ Everest *et al.* (2012b); ² Everest *et al.* unpublished.

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585 ³10% (n=48), adults were 14% (n=28) and juveniles 5% (n=20)

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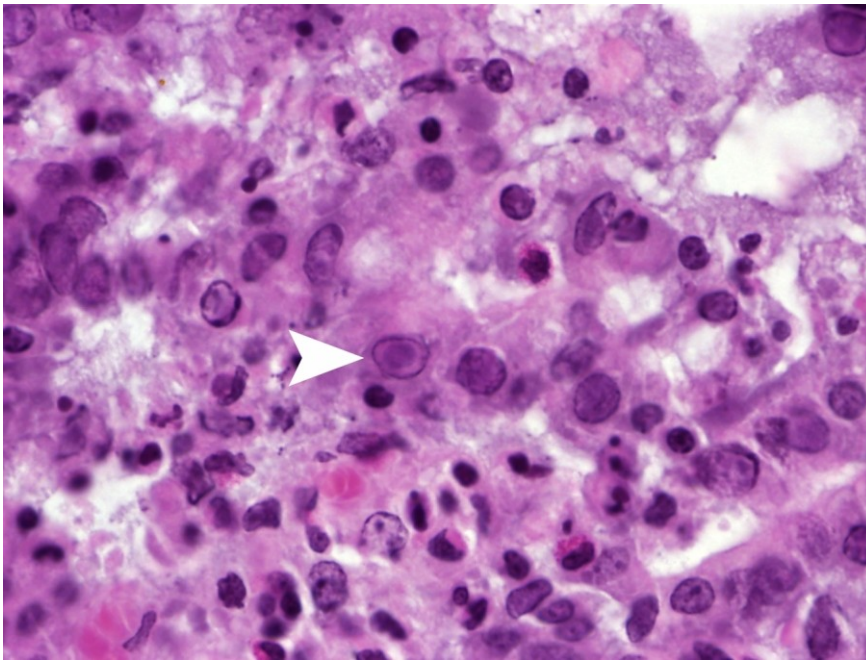
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592 **Figure 1.** Haematoxylin and Eosin histology image of a section of red squirrel
593 *Sciurus vulgaris* small intestine, showing intra-nuclear virus inclusion bodies
594 (arrowed) and extensive damage to the villi, findings consistent with adenovirus
595 infection. x600 magnification.

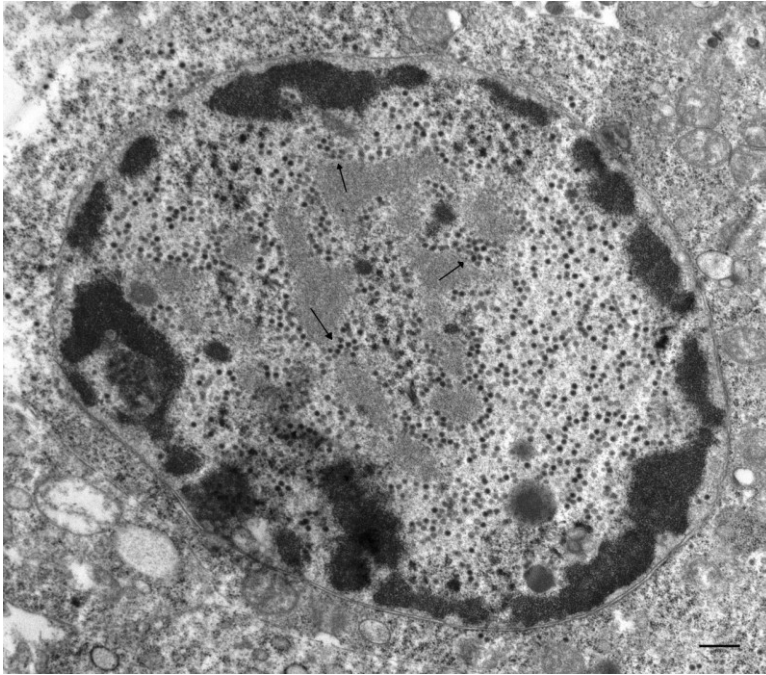


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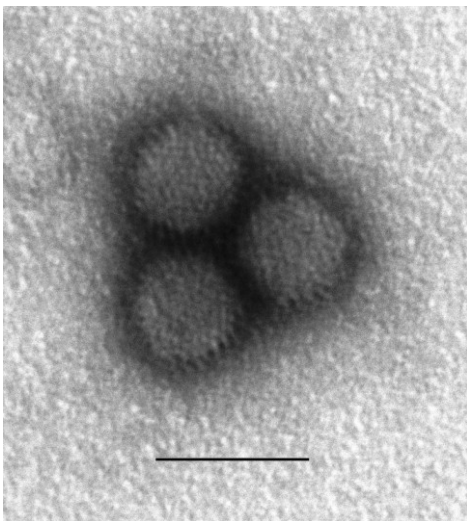
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599 **Figure 2.** Micrograph of adenovirus particles (arrowed) detected in an ultra-thin
600 section of enterocytes from a red squirrel *Sciurus vulgaris* large intestine. Bar
601 (bottom right) = 500 nm.



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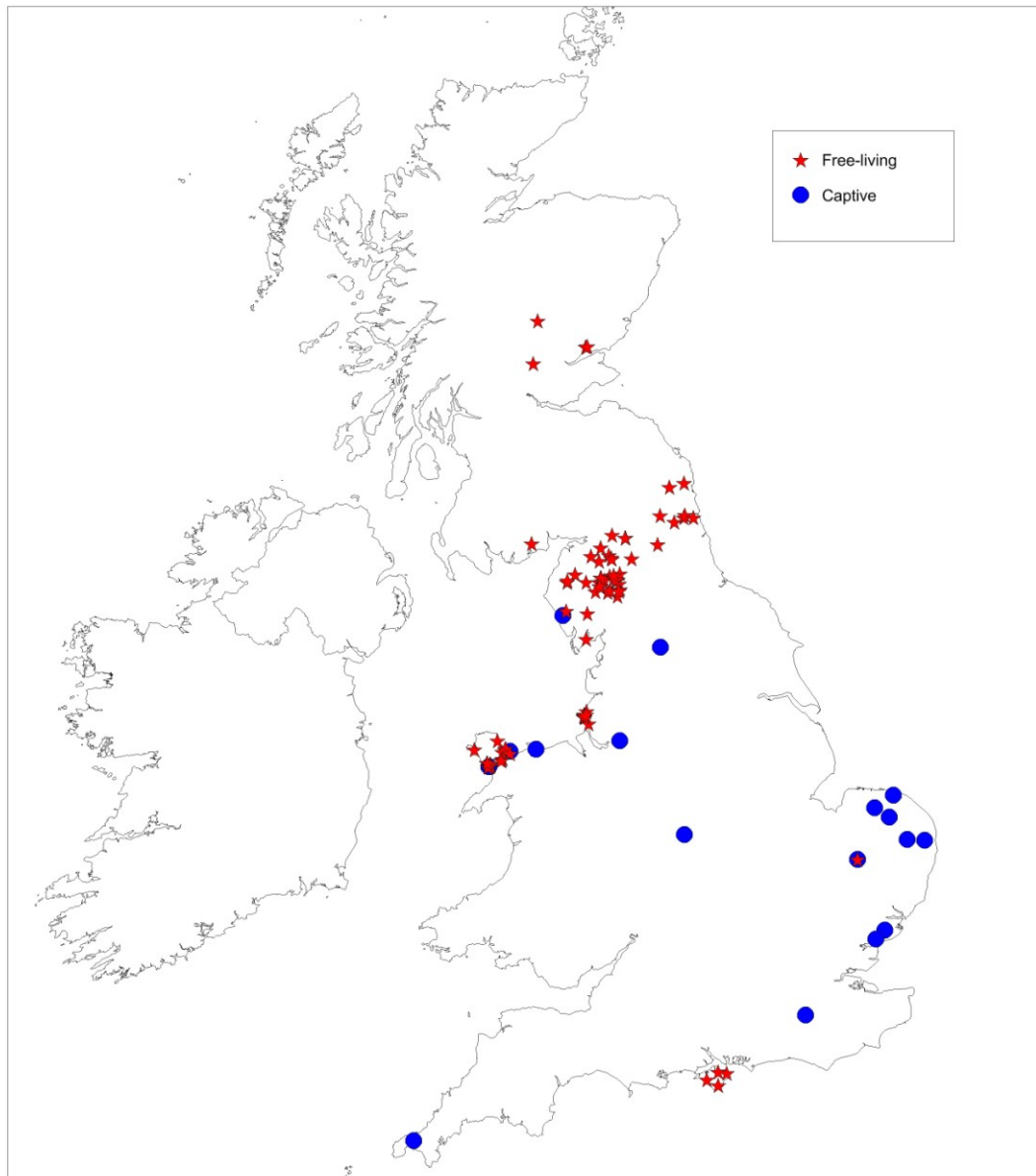
603 **Figure 3.** Micrograph of adenovirus particles detected in contents from the large
604 intestine of a captive red squirrel *Sciurus vulgaris*. Bar = 100nm.



605

606 **Figure 4.** Location of adenovirus positive free-living wild (●) and captive (□) red
607 squirrel *Sciurus vulgaris* cases from Great Britain, as analysed by PCR and TEM.
608

All cases of adenovirus in red squirrels in the UK to June 2013



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