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Original investigation

The implications of significant adenovirus infection in UK captive red squirrel (*Sciurus vulgaris*) collections: How histological screening can aid applied conservation management

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ABSTRACT

Conservation *trans*-locations using captive bred red squirrels (*Sciurus vulgaris*) is increasing in the United Kingdom (UK). However, project managers are often unaware of the risk of pathological adenovirus (ADV) infection. In this study we illuminate the viral threat using transmission electron microscopy (TEM) and polymerase chain reaction (PCR) assays. Both techniques were used to screen samples collected from 26 English and Welsh captive red squirrel collections. Of 181 carcasses received between 2002 and 2016, 129 (71%) were suitable for routine surveillance post mortem examination (PME). A range of tissues were examined with ADV identified from a variety of samples by PCR and TEM in 92 (72%) cases encompassing 23 of the 26 study collections (89%). ADV enteritis was histologically confirmed in two deaths (2%) with another 39 (30%) through both laboratory and clinical findings, considered as likely clinically-significant ADV cases, but advanced autolysis precluded accurate assessment and confirmatory histological diagnosis. Other positive cases were more indicative of sub-clinical infection. Clusters of ADV red squirrel deaths were recorded with circumstantial evidence suggesting inter-collection movement of presumed ADV infected donated animals had triggered mortality in recipient collections. During the study, several collections intermittently experienced ADV-associated deaths. Definitive cause of death was not determined in most cases, but a diverse range of diagnoses were recorded in 25 (19%) animals. Implications of these findings for captive United Kingdom (UK) red squirrel husbandry are discussed. It is recommended that protocols be drawn up to minimise potential intra-species ADV infection and highlight the danger of contact with ADV infected wood mice (*Apodemus sylvaticus*).

Introduction

In the United Kingdom (UK), the geographic range of the native red squirrel (*Sciurus vulgaris*) has dramatically declined, associated with habitat loss and woodland fragmentation. However, the most important factor in recent decades has been the steady expansion of and competitive replacement by the introduced North American Eastern grey squirrel (*Sciurus carolinensis*), (Gurnell et al., 2008, 2015). This invasive squirrel species is also a sub-clinical reservoir host of squirrelpox virus (SQPV), known to cause epizootic dis-

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ease in red squirrels (Rushton et al., 2006; Bruemmer et al., 2010; Chantrey et al., 2014). Growing evidence shows that adenovirus (ADV) can also cause significant red squirrel mortality (Everest et al., 2014), with a predilection for the intestinal mucosa, producing characteristic viral inclusions in infected villous enterocytes (Duff et al., 2007). Although sporadic ADV infection in UK grey squirrels has been recorded by polymerase chain reaction (PCR), surveillance for ADV-associated disease in the species by both PCR and transmission electron microscopy (TEM) has so far been minimal, with low level ADV presence (Everest et al., 2009) but no detectable mortality observed. The relative importance of inter- and intra-species ADV infection pathways in both sciurid species remains unclear.

Retrospective assessment of mortality in free-living wild red squirrels has identified sporadic clinically-significant cases with likely ADV involvement in several UK areas (Everest et al., 2010a, 2010b, 2012a). ADV has been recorded in both captive and free-ranging red squirrel populations associated with re-introduction programmes on Anglesey, northwest Wales (Everest et al., 2008, 2012b; Shuttleworth et al., 2016) and in an earlier 1990s Thetford Chase, Suffolk *trans*-location and release programme (Martinez-Jiménez et al., 2011). Because red squirrel re-introduction programmes rely on the use of Convention on International Trade in Endangered Species (CITES)-defined "bred in captivity" stock, or alternatively, involve licensed capture and *trans*-location of wild born animals, it is imperative that both reactive and proactive management of potential pathogens (see IUCN/SSC, 2013) form part of contingency planning.

Building upon earlier preliminary findings, Everest et al. (2014, 2015), produced evidence of more widespread ADV infection in red squirrels, including clinical and gross pathological features typically associated with disease caused by ADV infection. However, general carcass preservation, degree of autolysis and limited analytical testing combined to preclude definitive diagnoses in most cases. This subsequent surveillance study attempted to quantify the frequency of ADV-associated deaths within 26 captive red squirrel collections involving 22 English captive breeding or rehabilitation collections and four Welsh sites related to animals housed as part of red squirrel re-introduction programmes. Despite extensive pathological investigations, the underlying cause of death was not established in many cases due to advanced autolysis. An enteropathy was often suspected grossly at post mortem examination (PME), but frequently could not be subsequently confirmed histologically. Within this context and its practical limitations, we discuss the overall significance of ADV infection.

Material and methods

Post mortem examination and sample selection

Mortality records between 2002 and 2016 in the 26 captive red squirrel collections were collated. Of 181 carcasses submitted, 129 (71%) were considered adequately preserved for PME at six UK veterinary pathology laboratories: International Zoo Veterinary Group, Yorkshire, the Animal and Plant Health Agency (APHA) laboratories at Penrith, Starcross, York and Weybridge under the Diseases of Wildlife Scheme (*DoWS*) and the University of Liverpool. The number and range of tissues collected at PME was influenced by the degree of autolysis present. Histological examination was attempted if carcass preservation allowed, with more limited tissue harvesting undertaken on the more compromised submissions. Formalin-fixed paraffin-embedded and ultra-frozen tissues collected at these PMEs, together with other samples not specifically targeting ADV, but archived between 2002 and 2016, were retrieved and stored centrally at APHA-Weybridge. In addition, a range of unfixed tissues, including spleen, liver, stomach, intestine and faeces were retained from the PMEs and frozen at −70 °C. Intestinal samples where possible were available for retrospective testing for ADV by both TEM and PCR assays, but spleen or liver samples were limited to PCR assays. The total sample pool yielded 87 intestinal content/faecal or stomach content, 71 tissue samples (spleen, liver, intestine) and 11 paraffin embedded tissue blocks (PETBs) from 129 individual autopsied red squirrels. Matched faecal and tissue samples were obtained from 39 individuals.

Negative contrast stain TEM and PCR analyses

Previously described methodologies were used for the negative stain TEM (Everest et al., 2010b) and PCR assays (Everest et al., 2012a,b).

Ultra-thin section TEM

Large intestine sections were fixed in 3% glutaradehyde prepared in a 0.1 M phosphate buffer (pH7.4). Selected tissues were blocked to one to two mm in thickness for TEM examination. The tissues were washed in 0.1 M phosphate buffer, post fixed in 1% osmium tetroxide in phosphate buffer, dehydrated through a gradual series of ethanol solutions from 30 to 100% and placed in propylene oxide prior to embedding in araldite resin moulds. The resin was then polymerised at 60 °C for 48 h. One-micron sections, stained with toluidine blue, were prepared for light microscopy examination and areas with viral inclusions selected for ultrastructural examination. Ultra-thin sections cut at 90 nm thickness were then prepared onto 100 u copper grids using a diamond knife, contrasted with uranyl acetate and lead citrate prior to examination using an FEI TECNAI Bio-Twin 12 TEM.

Statistical analysis

The Chi- square test with Yates continuity correction was used to compare the frequency of ADV infection between sexes and ages. Logistical regression was utilised to determine any relationship between ADV infection and bodyweight. All analyses were conducted in R (R Core Team, 2015).

Results

Post mortem examination, histopathological findings and cause of death

In the majority of cases subjected to PME, the cause of death was unclear. A definitive diagnosis was determined in only 25 of 129 PME cases (19%), attributable to a diverse range of infectious and non-infectious conditions (Table 1), including two histologically confirmed ADV cases. A combination of excessive autolysis, no history including clinical signs and inadequate PME data precluded a diagnosis in 28 further submissions (22%). In the remaining 76 animals (59%), non-specific gross changes in the gut suggestive of an enteropathy were noted. Histological examination was frequently unrewarding, with an absence of significant lesions in non-intestinal organs and uninterpretable autolytic intestinal sections. Carcasses frequently displayed evidence of diarrhoea, with perineal faecal staining, often accompanied by soft or watery enteric content, occasionally blood stained, and scant residual rectal faeces. Two cases of ADV enteritis were confirmed histologically in well preserved intestinal tissue sections, with pathognomonic intranuclear viral inclusions and marginated nuclear chromatin in infected villous enterocytes, associated with mucosal necrosis, villous atrophy, mixed lymphocyte and plasma cell infiltrations of

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Table 1
Cause of death for 101 of 129 red squirrel post mortem examination cases.

Cause of death	Cases	Cause of death	Cases
Enteropathy (cause unknown)	76	Coccidiosis	1
Starvation/Malnutrition	8	Drowning	1
Other viral causes[a]	4	Fungal necrotizing enteritis[d]	1
Neoplasia[b]	3	Ivermectin toxicity	1
ADV enteritis	2	Myocarditis	1
Bacterial Infection[c]	2	Renal disease	1

A cause of death was precluded in the remaining 28/129 animals due to excessive autolysis (11), no observable lesions (10) or no recorded PME data (7).

[a] Recorded as death caused by viral infections, with particles detected by TEM, SQPV (1), Rotavirus (1), Enterovirus-like agent (1), Rota/Reovirus (1).

[b] Lymphosarcoma (2), Strangulated lipoma (1).

[c] *Streptococcus spp.* (1)*Staphylococcus sciuri* (1).

[d] Mucor/Absidia-like organisms.

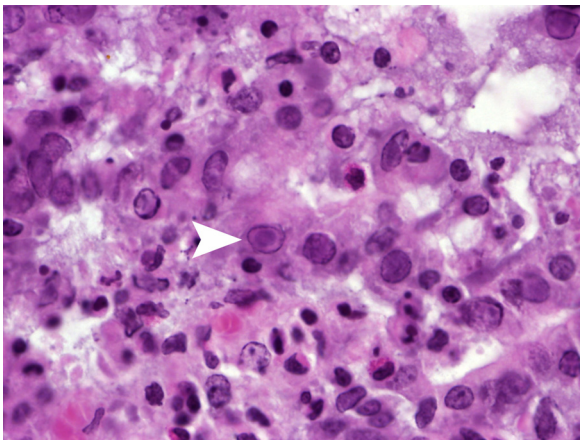


Fig. 1. ADV intranuclear inclusion body (arrowed) detected in large intestine tissue from a captive red squirrel. x600 Mag. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the lamina propria and submucosa and crypt hyperplasia with cell debris in gland lumina (Fig. 1). Multifocal splenic necrosis was often present, concentrated in germinal centres of white pulp, with occasional liver ADV inclusions detected (not shown).

ADV sample testing by TEM and PCR analyses

The ADV positives and each assay platform test numbers for all 129 tested carcasses from the 26 collections are presented in Table 2. ADV was detected in 92/129 (72%) of cases. Of these, 21/87 (24%) samples were positive when analysed by TEM (Fig. 2) and 71/107 (67%) samples were positive when analysed by PCR. Altogether, 65 animals had samples assayed by both procedures, with all animals negative by TEM on faecal material samples, but, 23/39 (59%) of the available spleens were positive for ADV by PCR. Additionally, 18/24 (75%) of TEM negative faecal material samples were also PCR positive for ADV. In all, of the 71 PCR positive cases, initial TEM screening failed to detect ADV particles in available intestinal or stomach content in 54 (76%) cases. Assay specificity was confirmed by PCR amplicon sequencing of five ADV positive cases detected between 2005 and 2006 from collection B (Fig. 3). Nucleotide sequences from first and second round products shared 100% identity with a 2007 UK free-living wild red squirrel ADV isolate (JN205244.1).

From the 129 carcasses, available PME report details identified 90 animals (70%) with an enteropathy (e.g. diarrhoeic, abnormally soft faeces or reddening of intestinal mucosa), 74 being ADV-associated infection cases, including all 21 TEM positive animals. Of the remaining 39 animals not exhibiting an enteropathy, 18

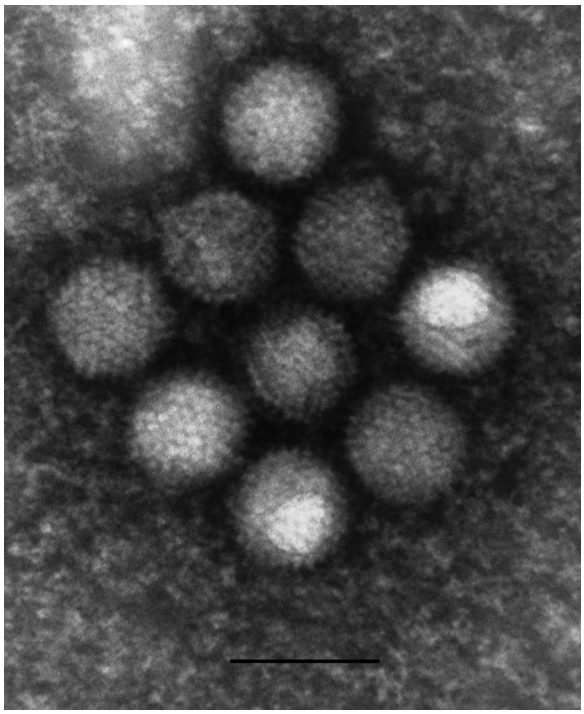
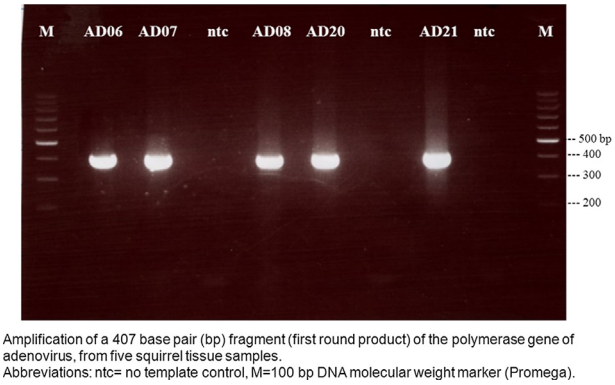


Fig. 2. Micrograph of ADV particles detected in a large intestinal faecal content sample from a captive red squirrel. *Bar = 100 nm.* (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



Amplification of a 407 base pair (bp) fragment (first round product) of the polymerase gene of adenovirus, from five squirrel tissue samples.
Abbreviations: ntc= no template control, M=100 bp DNA molecular weight marker (Promega).

Fig. 3. PCR agarose gel exhibiting amplified ADV DNA bands detected in spleen and liver tissue from five captive red squirrels. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were ADV positive by PCR analyses. For the 39 animals providing matched faecal material and spleen tissue samples, 23/39 (59%) were positive for AVD on spleen tissue by PCR, but all were TEM negative on available faecal material samples. In addition, three cases were detected with non – ADV virus particles in faecal matter by TEM. The first one involved an enterovirus agent, an index case in Britain for the species, the second one a rotavirus and the third presented a dual infection involving both rotavirus and reovirus, another index case. In our experience, in faecal material, TEM only detects these enteric virus particles just prior to death and are, combined with enteric abnormality and diarrhoeic presence, regarded as the likely cause of death. A fourth viral agent was SQPV, with particles detected by TEM from typical SQPV skin lesion material and all four cases are denoted in Table 1. Two of these four animals were also ADV positive by PCR. The combined ADV results from all 26 collections are shown geographically, giving locations for the 23

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Table 2
ADV-associated deaths and tests for each assay type utilised for the 129 examined carcasses across the 26 UK study collections.

Collection	ADV Positive/Tested	Collection	ADV Positive/Tested
A	24/36	N	1/1
2002 to 2016	[1] (23/35) 68% [2] (1/20) 5%	2011	[1] (1/1) 100% [2] (0/1) 0%
B	11/12	O	0/2
2004 to 2007	[1] (8/9) 89% [2] (3/3) 100%	2012 to 2013	[1] (0/2) 0% [2] (0/2) 0%
C	1/1	P	1/1
2004	[1] (1/1) 100%	2012	[1] (1/1) 100% [2] (0/1) 0%
D	1/1	Q	3/3
2005	[1] (1/1) 100%	2012	[1] (3/3) 100% [2] (0/3) 0%
E	1/1	R	3/3
2006	[1](1/1) 100%	2012 to 2014	[1] (3/3) 100% [2] (0/1) 0%
F	1/2	S	2/5
2007 to 2009	[2] (1/2) 50%	2013 to 2014	[1] (2/5) 40% [2] (0/2) 0%
G	1/1	T	1/2
2007	[1] (1/1) 100% [2] (0/1) 0%	2013	[1] (1/2) 50% [2] (0/2) 0%
H	11/17	U	1/1 (100%)
2009 to 2016	[1] (5/11) 46% [2] (6/13) 46%	2013	[1] (1/1) 100% [2] (0/1) 0%
I	12/14	V	3/3
2010 to 2015	[1] (7/9) 78% [2] (5/13) 39%	2013	[1] (3/3) 100% [2] (0/3) 0%
J	1/3	W	1/5
2010 to 2013	[1] (0/2) 0% [2] (1/3) 33%	2014 to 2016	[1] (1/5) 20% [2] (0/1) 0%
K	3/3	X	4/5
2011 to 2016	[1] (1/1) 100% [2] (2/3) 67%	2015 to 2016	[1] (4/5) 80% [2] (0/5) 0%
L	2/2	Y	0/1
2011	[1] (1/1) 100% [2] (1/2) 50%	2015	[1] (0/1) 0% [2] (0/1) 0%
M	0/1	Z	3/3
2011	[1] (0/1) 0% [2] (0/1) 0%	2016	[1] (2/2) 100% [2] (1/3) 33%

A to Z: ADV positives 92/129 animals (72%): [1](71/106), [2](21/86) samples.
[1]PCR analyses, [2] TEM analyses.

ADV positive and three ADV negative collections by the combined detection methods (Fig. 4).

Statistical analysis

There were no statistically significant differences in ADV infection between age, sex or bodyweight of the squirrels.

Discussion

This study is the only one of its kind undertaken to investigate ADV infection presence within UK captive red squirrel collections, and how its presence may affect the management of such collections within the UK captive red squirrel breeding scheme and its associations with re-introduction and *trans*-location exercises in order to re-inforce the remaining UK red squirrel population. Although technical in nature, which is of necessity, the study findings are of paramount importance to the conservation of the species in this context, as they illuminate a number of concerns that would, given the opportunity, affect the outcome of such re-enforcement programmes. ADV infection was detected at 23 of the 26 collections tested, (19 of the English permanent and rehabilitation collections and all four Welsh sites associated with re-introductions. These data indicate ADV is more geographically widespread than previously documented free-ranging and captive red squirrel studies suggest (Everest et al., 2010b, 2012b). These findings have implications for future captive animal stock management and *trans*-location programmes. A previous study (Everest et al., 2010b), using TEM as the sole assay in animals specifically selected for enteric abnormalities, found 14% (10/70) of free-living red squirrel carcasses found dead and submitted for examination to be ADV positive. Given the earlier paucity of data on ADV infection in UK red squirrels, the disease, until recently (Everest et al., 2008, 2012b), was not considered a particular threat to captive populations, with targeted testing not being undertaken. In contrast, in our study, enteropathy was not a case selection criterion and crucially, specific ADV PCR analyses were used in conjunction with TEM.

With this dual testing, evidence of ADV infection was identified in 72% (92/129) of the carcasses examined, in 21/87 (24%) by TEM and in 71/107 (67%) by PCR (Table 2). The use of viral particle detection when using TEM is essentially restricted to animals displaying enteric abnormalities such as those described above and as previously discussed, particle detection in faecal material will in our experience be restricted to detection in animals just prior to death. PCR on the other hand offers a much wider scope for viral infection detection, as due to the higher analytical sensitivity, will detect viral DNA at far lower concentrations than TEM could detect particles. In this way, sub-clinical infections may be detected in animals which had died of other causes and which would not ordinarily have been seen. With this dual assay platform in operation, clinically-significant cases such as the case described in Figs. 1, 2 and 5 may therefore be detected alongside the 54 TEM negative animals which were ultimately PCR positive. However, while PCR is evidently more reliable at detecting sub-clinical ADV-associated infection, the 'open view' nature of TEM allows a wider range of viruses to be detected in a sample as seen in Table 1. Combining both clinical histories and PME findings, 90 of 129 carcasses (70%) had an enteropathy, with 74 testing ADV positive. All 21 ADV TEM positive animals presented with an enteropathy and of the 39 animals without enteric signs, 18 were detected positive for ADV infection presence by PCR.

The ADV infection rate, (clinically-significant and likely sub-clinical infection), associated with red squirrel mortality was higher in captive animals in close contact within enclosures, so aiding ADV spread, compared to free-living animals, (findings similar to Everest et al., 2012a,b). Contact with wood mice (*Apodemus sylvaticus*), potentially infected with murine ADV, has been mentioned as a potential factor to increase grey squirrel ADV infection rates, by facilitating (as yet unconfirmed) inter-species transfer, (Greenwood and Sanchez, 2002; Everest et al., 2013), which may in some cases possibly lead to ADV presence in sympatric red squirrel populations. All study sites contained areas of woodland (mainly deciduous) and at two locations (collections A and H), wood mice were trapped inside red squirrel enclosures for sampling. PCR anal-

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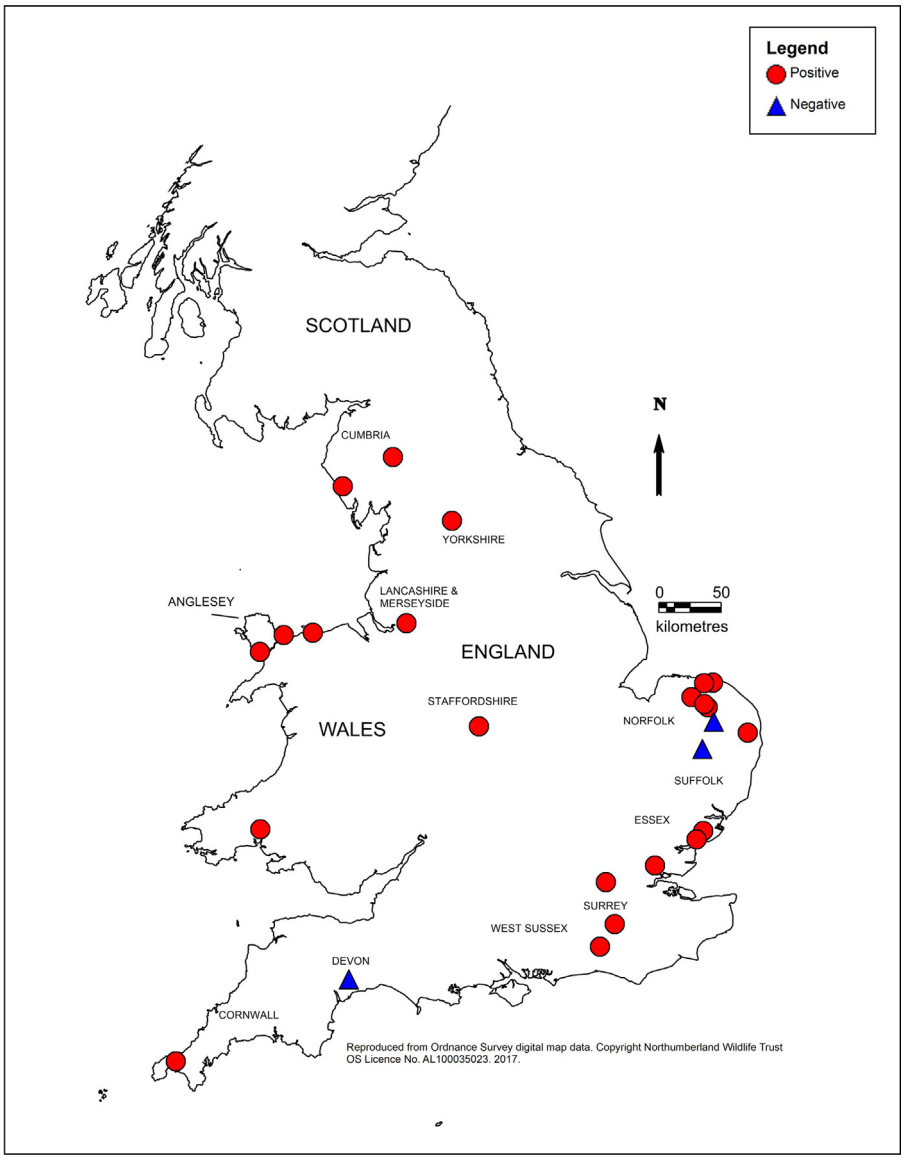

Fig. 4. Positive and negative ADV results for each geographic location for the 26 captive red squirrel collections studied, analysed by PCR and/or TEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

yses on spleen tissue subsequently detected 2/24 (8%) wood mice positive for ADV at collection A (Everest et al., 2015). Earlier advice, regarding active mouse control in red squirrel enclosures (Everest et al., 2014), is still a prudent measure, until the epidemiology of ADV in mice, transmission pathways and associated risks posed to red squirrels by wood mice can be more accurately determined.

Everest et al. (2014), previously suggested that advanced autolysis is liable to adversely affect or even destroy significant gross and histopathological changes, particularly in the intestinal tract. Insufficient PME data effectively precluded any meaningful investigation into 28 of the study carcasses by both PCR and TEM analyses. Histological examination of fixed tissues, especially intestine, was frequently unrewarding, but should always form part of any detailed mortality study protocol, to help aid result interpretation. Definitive cause of death from post mortem examination and associated analytical testing were only determined in 25 animals (Table 1), but included two unequivocal clinically-significant ADV enteritis cases with pathognomonic histopathological features in the intestinal mucosa, one case represented in Fig. 1 as displaying inclusion bodies within intestinal mucosal tissue on a Haemotoxylin and Eosin slide, followed by ultra-thin sectioning to reveal intracellular ADV viral particles in Fig. 5. Both of these animals were only analysed by TEM.

Based on our study data, we believe that unequivocal active ADV infection confirmation requires a prompt PME of submitted carcasses within a few hours of death to identify intact pathognomonic intranuclear inclusion bodies in infected villus enterocytes (Duff et al., 2007), supported if necessary by TEM viral particle detection in ultra-thin intestinal sections (Fig. 5). These exacting time limitations create logistical difficulties, particularly with captive animals which may spend extensive periods unobserved in nest boxes. Furthermore, identifying individual clinical cases is difficult when animals are housed in groups, due to similar body size or pelage characteristics (Shuttleworth et al., 2009). Significant delays in carcass discovery and retrieval from nest boxes was inevitably associated with confounding advanced autolysis resulting in 52 carcasses (29%) considered as unsuitable for PME. Of the 129 (71%) better preserved cases examined, although no significant differences in ADV infection were found between age, sex and squirrel bodyweight, the number of cases seen in females and juveniles

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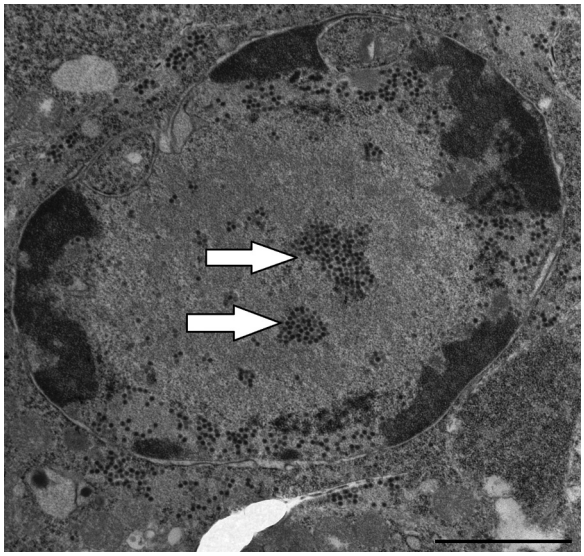

Fig. 5. Ultra-thin section of large intestine enterocytes from a captive red squirrel exhibiting ADV particles. *Bar = 2um*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
ADV-associated cases in red squirrels detected at recipient study collections following receipt of stock from other captive study collections.

Collection	Donor collection	ADV infection	Stock receipt	ADV year
A	Q	Yes	2005	2005
B	Q	Yes	2005	2005
E	N	Yes	2006	2006
H	R	Yes	2009	2009
J	H	Yes	2013	2013
L	H	Yes	2011	2011
N	I	Yes	2011	2011
S	I	Yes	2013	2013
U	I	Yes	2014	2015
V	I	Yes	2013	2013
X	I	Yes	2015	2015
Z	I	Yes	2015	2016

is most likely a reflection of closer confines within nest boxes between mothers and kittens prior to leaving the box, facilitating wider ADV spread. On occasions, in mortality clusters where no material was made available for analysis, initial clinical observations at autopsy had suggested a potential for ADV involvement, but the lack of analytical material precluded a definitive diagnosis, viral or otherwise.

Inter-collection animal movement, associated with stock mortality provided good circumstantial evidence for direct captive animal-to-animal transmission of ADV infection. Table 3 denotes 12 collections which had been detected with ADV infection presence following receipt of donor stock from other known ADV infected study collections. Three such ADV scenarios have been described in detail, highlighting the ADV risk in each case. Eleven recorded deaths at collection A during 2005 and 2006 were of quarantined donor stock (from collection Q), held prior to transfer to other locations. The severity of this prolonged retrospectively confirmed ADV infection outbreak highlights the risks posed by ADV to a UK captive red squirrel collection. Data from 2009 (collection H) reported six ADV-associated infection cases following donor stock arrival from collection R, aimed at enhancing the recipient collection's genetic diversity. After arrival, to minimise handling stress when collecting biometric data and reading Passive Integrated Transponder (PiT) tags, the caged quarantined juvenile red squirrels were moved from their shed to a larger open air enclosure, containing resident red squirrels on public display. After weighing and sexing, they were re-caged and returned to their quarantine shed. Subsequently, the four donor animals within their shed, a resident from the display enclosure and another red squirrel housed in an adjacent connected enclosure developed clinical diarrhoea and died and active ADV infection was confirmed by faecal TEM analyses. Investigations revealed sub-optimal hygiene standards with shared cleaning equipment and poor equipment disinfection procedures, all predisposing to ADV spread between groups. Determining conclusively whether the infection had been pre-existent or introduced by inter-collection movement was compromised by minimal pre-movement ADV monitoring.

To contrast this event and the earlier outbreak at collection A, both coinciding with donor stock arrival, 11 deaths between 2010 and 2011 were recorded at collection I (Table 2), where no recent animal introductions had occurred and no obvious ADV squirrel source was identified, so other potential incursion routes such as murine ADV infected wood mice presence have to be considered. Since this initial 2010 outbreak, stock from this collection was subsequently moved to seven known other captive or ultimately re-introduced wild populations, all of which were included in this study (collections N, S, U, V, X, Y and Z), where evidence of ADV infection from both TEM and PCR analyses in red squirrels has since been detected at six study sites (collections N, S, U, V, X and Z; Table 3). Evidence of diarrhoea was present in each collection, but delayed detection of mortalities allied with excessive autolysis again precluded confirmatory histological confirmation of ADV enteritis. On average, infection in recipient collections manifested itself within a few weeks or months of receipt. Adenovirus therefore presents a substantial threat to the success of initiatives to re-inforce native red squirrel populations across its UK range. Discussions between collections regarding whether all donor stock is free from ADV infection needs to be undertaken, as our results clearly show this is not the case, but careful management of those that are, within the UK captive breeding scheme with stringent pre-movement virus testing and diligent biosecurity measures should help to achieve this.

To conclude, although our study is primarily a mortality and infection study, our findings are critical to the conservation of the red squirrel in the UK via re-introduction and *trans*-location studies, which are used to supplement the current population. Standard protocols to mitigate potential for infection spread among their collections should be provided, ideally based on robust quarantine facilities, effective hygiene measures, rodent control and appropriate pre-movement animal screening at the collection's discretion, involving ADV PCR and/or TEM analysis of faecal material to ensure all ADV cases are detected within the captive collections. Such procedures will have a financial implication, but should ultimately improve the outlook for the native red squirrel by reducing mortality and improving stock welfare in captive collections, so proving cost effective in the longer term.

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References

Bruemmer, C.M., Rushton, S.P., Gurnell, J., Lurz, P.W.W., Nettleton, P., Sainsbury, A.W., Duff, J.P., Gilray, J., McInnes, C.J., 2010. Epidemiology of squirrelpox virus in grey squirrels in the UK. Epidemiol. Infect. 138, 941–950.

Chantrey, J., Dale, T.D., Read, J.M., White, S., Whitfield, F., Jones, D.N., McInnes, C.J., Begon, M., 2014. European red squirrel population dynamics driven by squirrelpox at a gray squirrel invasion interface. Ecol. Evol. 4, 3788–3799.

Duff, J.P., Higgins, R., Farrelly, S., 2007. Enteric adenovirus infection in a red squirrel (*Sciurus vulgaris*). Vet. Rec. 160, 384.

Everest, D.J., Stidworthy, M.F., Shuttleworth, C.M., 2008. Adenovirus-associated mortalities in red squirrels (*Sciurus vulgaris*) on Anglesey. Vet. Rec. 163, 430.

Everest, D.J., Grierson, S.S., Stidworthy, M.F., Shuttleworth, C.M., 2009. PCR detection of adenovirus in grey squirrels on Anglesey. Vet. Rec. 165, 482.

Everest, D.J., Grierson, S.S., Meredith, A.M., Milne, E.M., 2010a. Adenovirus in a red squirrel (*Sciurus vulgaris*) from Scotland. Vet. Rec. 167, 184.

Everest, D.J., Stidworthy, M.F., Milne, E.M., Meredith, A.L., Chantrey, J., Shuttleworth, C.M., Blackett, T., Butler, H., Wilkinson, M., Sainsbury, A.W., 2010b. Retrospective detection by negative contrast electron microscopy of faecal viral particles in wild red squirrels (*Sciurus vulgaris*) with suspected enteropathy in Great Britain. Vet. Rec. 167, 1007–1010.

Everest, D.J., Griffin, J., Warnock, N.D., Collins, L., Dick, J., Reid, N., Scantlebury, M., Marks, N., Montgomery, I., 2012a. Adenovirus particles from a wild red squirrel (*Sciurus vulgaris*) from Northern Ireland. Vet. Rec. 170, 188.

Everest, D.J., Shuttleworth, C.M., Grierson, S.S., Duff, J.P., Jackson, N., Litherland, P., Kenward, R.E., Stidworthy, M.F., 2012b. A systematic assessment of the impact of adenovirus infection on a captive re-introduction project for red squirrels (*Sciurus vulgaris*). Vet. Rec. 171, 176–182.

Everest, D.J., Butler, H., Simpson, V.R., Blackett, T., Shuttleworth, C.M., 2013. Adenovirus infection in red squirrels in areas free from grey squirrels. Vet. Rec. 173, 199–200.

Everest, D.J., Shuttleworth, C.M., Stidworthy, M.F., Grierson, S.S., Duff, J.P., Kenward, R.E., 2014. Adenovirus: an emerging factor in red squirrel *Sciurus vulgaris* conservation. Mamm. Rev. 44, 225–233.

Everest, D.J., Shuttleworth, C.M., Grierson, S.S., Dastjerdi, A., Stidworthy, M.F., Duff, J.P., Sainsbury, A.W., 2015. The impact of adenoviruses on red squirrel (Sciurus vulgaris) conservation ?with notes on other enteric viruses of potential significance. In: Shuttleworth, C.M., Lurz, P.W.W., Hayward, M. (Eds.), Red Squirrels: Ecology, Conservation and Management in Europe. European Squirrel Initiative, Suffolk, pp. 129–146.

Greenwood, A.G., Sanchez, S., 2002. Serological evidence of murine pathogens in wild grey squirrels (*Sciurus carolinensis*) in North Wales. Vet. Rec. 150, 543–546.

Gurnell, J., Lurz, P.W.W., Halliwell, E., 2008. Red squirrel (Sciurus vulgaris). In: Harris, S., Yalden, D.W. (Eds.), Mammals of the British Isles. Handbook. , 4th edition. The Mammal Society, Southampton.

IUCN/SSC, 2013. Guidelines for Reintroductions and Other Conservation Translocations. Version 1.0, viiii. IUCN Species Survival Commission, Gland, Switz, pp. 57.

Martinez- Jiménez, D., Graham, D., Couper, D., Benko, M., Schoniger, S., Gurnell, J., Sainsbury, A.W., 2011. Epizootiology and pathologic findings associated with a newly described adenovirus in the red squirrel, (*Sciurus vulgaris*). J. Wildlife Dis. 47, 442–454.

2015. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria (URL) http://www.R-project.org/.

Rushton, S.P., Lurz, P.W.W., Gurnell, J., Nettleton, P., Bruemmer, C., Shirley, M.D.F., Sainsbury, A.W., 2006. Disease threats posed by alien species: the role of a poxvirus in the decline of the native red squirrel in Britain. Epidemiol. Infect. 134, 521–533.

Shuttleworth, C.M., Kenward, R.E., Jackson, N., 2009. The reintroduction of the red squirrel Sciurus vulgaris to Newborough forest, North Wales: a five year project review. In: Report to Countryside Council for Wales. Wildlife Trust, Forestry Commission Wales and Grantscape.

Shuttleworth, C.M., Kenward, R.E., Jackson, N.J., 2016. Developing red squirrel re-introduction techniques for use during regional grey squirrel eradication programmes in Europe. In: Soorae, P. (Ed.), Global Re-introduction Perspectives: 2016 Case-studies from Around the Globe. IUCN/SSC Re-introduction Specialist Group, pp. 182–189.

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