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Research Article

Inter-specific viral infections: Can the management of captive red squirrel collections help inform scientific research?

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Abstract

Squirrelpox virus (SQPV) and adenovirus produce pathological disease in native red squirrels (*Sciurus vulgaris*). SQPV in particular is a significant factor in regional population declines and is generally prevalent in the UK's introduced grey squirrel (*Sciurus carolinensis*) population as an asymptomatic infection. Despite the role of the grey squirrel as a virus reservoir and potential inter-specific infection pathways being highlighted, there remains a paucity of field study data with known relative inter-specific infection rates and quantified frequency of interactions. Intriguingly, whilst captive zoological red squirrel collections are often present within woodland habitat containing wild grey squirrels, clinical pox cases are rarely observed unless red squirrels are released from the enclosures. In 2011 we monitored grey squirrel activity on an enclosure containing red squirrels. Grey squirrels were present for a cumulative total of 47.5 minutes within the twenty four hours of observation. A range of behaviours were recorded including feeding, and instances where discarded food fell into the red squirrel enclosures below. We interpret the value of these observations in the context of published theories of viral transmission. The local grey squirrels were subsequently culled and tested for evidence of both historical and current SQPV and adenovirus infections. Polymerase Chain Reaction (PCR) assays did not amplify adenovirus DNA from grey squirrel blood samples, but positive results were recorded in faeces (3/18, 17%) and (10/18, 56%) in parallel spleen samples from the same animals. This variation in tissue specific detection rates suggests that previous long-term surveillance of adenovirus in wild grey squirrels focussing on blood samples may have significantly underestimated true infection rates. Enzyme-Linked Immunosorbent Assay (ELISA) tests revealed exposure to SQPV by antibody presence in 33% of the animals. Additionally, 22% of the animals contained detectable levels of both viruses. In parallel with laboratory and field studies in 2011, we collated historical unpublished reports and archived data from a range of UK squirrel collections and highlight some key cases of infection. We recommend that further behavioural and viral screening studies are focussed within scenarios where captive red squirrels are sympatric with wild grey squirrels.

Introduction

The grey squirrel (*Sciurus carolinensis*) has been highlighted as an example within the 100 worst global invasive alien species (Lowe et al., 2000). Since its introduction to the United Kingdom (UK) the species has caused extensive woodland damage by bark stripping (Huxley, 2003) and the regional extinction of native red squirrel (*Sciurus vulgaris*) populations through mechanisms including seed cache piracy and resource competition (Kenward and Holm, 1993; Wauters et al., 2000, 2002; Gurnell et al., 2001, 2004). In addition, grey squirrels generally carry squirrelpox virus (SQPV) as an asymptomatic infection. Inter-specific infection of sympatric red squirrels leads to epizootic disease in this species and rapid population declines (Thomes et al., 2003; Rushton et al., 2006; Sainsbury et al., 2008; Carroll et al., 2009; Bruemmer et al., 2010).

Adenovirus DNA has also been amplified from grey squirrels using Polymerase Chain Reaction (PCR) assays (Everest et al., 2009; Romeo et al., 2014); a virus that appears to be found as both asymptomatic and clinically-significant infections in wild red squirrels (Everest et al., 2012). For this particular virus, a paucity of research data means it remains unclear as to whether grey squirrels are an inter-specific viral reservoir, and to what extent, if any, inter-specific infection may involve other woodland rodents such as the wood mouse (*Apodemus sylvaticus*) (Everest et al., 2012). Further investigations are consequently a priority.

Red squirrel captive breeding programmes have played an important role in UK red squirrel conservation (Joint Nature Conservation Committee, 1996), and our understanding of pathogenic disease in particular has been advanced through re-introduction and trans-location studies involving these animals (Venning et al., 1997; Shuttleworth et al., 2008; Martínez-Jiménez et al., 2011; Everest et al., 2012, in press). A review of the literature will reveal however, that many of these adenovirus and squirrelpox data were obtained not through pre-planned proactive experimental scientific study, but reactively, with information gathered as the result of unexpected group mortality. Such reactive studies by their very nature may not facilitate future experimental repetition but they have offered an invaluable foundation for future research. In the absence of standardised experimental field-studies, Schuchert et

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al. (2014) have demonstrated the potential value of analysing what are often unbalanced and incomplete data sets produced within long-term practical conservation initiatives when data from more experimental studies are unavailable.

In their review of SQPV infection, Bruemmer et al. (2010) highlighted that there were limited quantified data on SQPV infection within captive red squirrel populations. In order to address this, we accessed unpublished reports and fragmentary archived material relating to captive red squirrels and associated release studies. The British and Irish Association of Zoos and Aquariums (BIAZA) provided information on 17 UK captive collections which indicated that the average collection held five adult squirrels (range 2-35 individuals) and animals were typically housed across 1-3 enclosures (Peter Litherland *unpublished data*). It became apparent that in at least four instances (Welsh Mountain Zoo, Wildwood, Pensthorpe and the Tilgate Nature Centre) captive Zoological red squirrel collections had been housed in proximity to wild grey squirrels for more than a decade without developing disease; so providing a unique and long-term experiment where spatial partitioning of red and grey squirrels had in some cases (Welsh Mountain Zoo and Tilgate Nature Centre) been punctuated by the release (or escape) of a sub-sample of the captive collection.

One significant study encompassed the trial release of captive-bred animals by the National Zoological Society of Wales in the late 1990s (Jackson, 1999). During this period, there was of course little understanding of SQPV and the extent to which inter-specific infection was a factor in red squirrel declines (Sainsbury et al., 1997; Schuchert et al., 2014). Thus, whilst rigorous health screening and monitoring protocols recognised the possible significance of diseases, the specific inter-specific threat posed by SQPV only became apparent as the Welsh project progressed. This is an important context, as today similar studies that did not seek to eradicate grey squirrels prior to red squirrel release would be unlikely to be given permission to be repeated and hence the historical data-sets have a novel and unique value.

Historically, two trial releases of captive bred and radio collared red squirrels were recorded at the Welsh Mountain Zoo, Colwyn Bay, North Wales. In 1996, during the first release, one adult male (Microchip number BDD0) was recaptured with lesions typical of squirrelpox, a suspicion later confirmed by both Transmission Electron Microscopy (TEM) and Enzyme-Linked Immunosorbent Assay (ELISA) analyses. The animal was hospitalised and treated for secondary infection. After 38 days it was judged to have made a full recovery and was returned to an outdoor enclosure from which it was subsequently re-released in 1997 along with three other captive bred red squirrels.

A review of veterinary records and archived viral test results held at Moredun Research Institute, Edinburgh, not only confirmed SQPV infections in the release programmes, but also revealed that in 2004, an escaped female red squirrel was recaptured at Welsh Mountain Zoo and found to have poxvirus antibodies. To date, other red squirrels tested at the site whilst in captivity have all been SQPV antibody negative even though grey squirrels can access the mesh frame construction of the squirrel exhibit enclosure. This evidence suggesting virus exposure was limited only to released animals formed the basis for a short field study in 2011. The research sought to quantify potential inter-specific infection pathways (e.g. grooming which may dislodge fleas, or other ectoparasites and discarded food falling into the enclosure which may have saliva present) by quantifying grey squirrel activity budgets and behaviour on red squirrel enclosures at Welsh Mountain Zoo and then through PCR and ELISA tests, assessing infection rates in the sympatric grey squirrels following culling. These results, along with archived evidence sourced from unpublished manuscripts

Figure 1 – The red squirrel release and captive breeding enclosures at Welsh Mountain Zoo 2011. The structure is little changed from that present in the late 1990s when re-introduction studies were undertaken.

and data-sets are combined to explore the potential risk that adenovirus and SQPV in grey squirrels pose to captive red squirrels. This study appears to be the only one of its type conducted in the UK and the results may help preclude some potential routes of transmission and provide data that may provide useful context for future studies seeking to focus upon other likely inter-specific infection pathways.

Study Site and Methods

In the spring of 2011 we reviewed a range of reports relating to mortality in captive red squirrels (e.g. Jackson 1999) and gathered information from popular publications e.g. Stapleford (2003). In parallel, we catalogued sporadic cases of squirrelpox relating to captive red squirrels in the UK using TEM data held at AHVLA-Weybridge and Moredun Institute, Edinburgh.

Where available, the results of ELISA tests from grey squirrels caught near captive red squirrel collections were quantified. Data were available from a limited range of geographical sites including Cumbria 1994 (SD103964), Surrey 1995 (SU813427), Suffolk 1999 (TL8183) and Staffordshire 2007 (SK195016). Red squirrel tissue and lesion material screened by TEM from a death within a trans-location study on Goathorn peninsula, Dorset (SZ013837) were also available. These tests were conducted several years after Kenward and Hodder (1998) published an account of the 1993 trans-location. The catalogue of historical data was examined to build a picture of squirrelpox in captive red squirrel populations in locations where grey squirrels were present close to red squirrel enclosures.

Comprehensive written records of two trial captive red squirrel releases undertaken at the Welsh Mountain Zoo, Colwyn Bay in the late 1990s were accessed and the results of historical ELISA SQPV antibody tests carried out on red and grey squirrels examined. Within the archived data-sets, red squirrel test results were sometimes, and confusingly, referenced against one of a number of different codes. Although the unique identification number associated with an individual captive animal was commonly listed, there were a small number of cases where these data were omitted and instead other submission codes were present. In these circumstances we retrospectively checked associated paperwork and were thus able to confirm animal identifications based on standardised Zoological stud book identification numbers.

The Welsh Mountain Zoo is set in 15 hectares of woodland and gardens in Colwyn Bay, Conwy County, North Wales, UK. Red squirrels are housed here in a series of inter-connecting enclosures covering approximately 200 m^2 (Fig. 1) Enclosure frames are constructed of 10×5 cm or 10×10 cm tantalised softwood timber with 2.5×2.5 cm square mesh wire sheeting forming a single skin wall or roof panel.

The red squirrel enclosure is built within a mixed deciduous and coniferous woodland with Ash (*Fraxinus excelsior*) Oak (*Quercus petraea*), Scots Pine (*Pinus sylvestris*) notable species. The enclosure typically houses 3-6 adult red squirrels and young are born annually. Grey squirrels are abundant in the Colwyn Bay area, and benefit from exploiting foods on garden bird tables. *Ad hoc* culling has taken place within the Zoo grounds, but wider landscape control is likely to be limited in the wooded gardens and residential grounds of Colwyn Bay. This has meant that re-invasion is rapid and hence a resident wild grey squirrel population is a continual presence within the Zoo.

Having established the historical pattern of SQPV infection within red squirrels released in the 1990s (see results), in the period 20th to 27th June 2011 (inclusive), the frequency and behaviour of grey squirrels active on red squirrel enclosures was quantified. Data were collected within one hour duration observation periods. We selected hours between 8am-11am and 3pm-7pm excluding mid-day when squirrels are generally inactive in the summer months (Gurnell, 1991). A cumulative observation total of 24 hours was recorded. The aim was not to examine temporal activity patterns but rather more simply to gauge what types of behaviours grey squirrels undertake when active and on the enclosures.

Grey squirrel behaviour was recorded using a basic scan sampling method. An animal's activity on the enclosure was recorded every 15 seconds using broad behavioural categories:
Travelling (moving rapidly across the construction), resting (sitting relatively motionless), grooming, feeding (on both natural and supplemental foods), searching (moving slowly across the construction deliberately searching the surface) were identified. When the animal was on the enclosure but a clear view was hampered by wooden posts was also recorded.

On two occasions when two grey squirrels were present together (15 s and 3 min 45 s respectively), data were collected for both animals separately.

Noteworthy observations were made including exploitation of spilt feed outside enclosures by grey squirrels and grey squirrel activity in the tree canopy overhanging the enclosures.

Following the cessation of behavioural observation studies, seven female and eleven male grey squirrels were trapped in wire treadle live capture "Mink" traps positioned in woodland between 5m and 150 m from the red squirrel enclosures. Captures occurred between 29/06/2011 and 05/07/2011. Squirrels were euthanized using the cranial dispatch method (Mayle et al., 2007). The mean body weight was 502.5 g (s.d. 85.78, n=18) and blood (2 ml), spleen and faecal samples were collected from each individual carcass.

We applied both PCR and negative contrast stain TEM to determine the presence of adenoviral DNA and viral particles respectively. The TEM methodology is previously described in Everest et al. (2010) and was applied to grey squirrel faecal samples only. The PCR assay methodology, described in detail in Everest et al. (2012), was applied to all grey squirrel material examined: faecal samples, spleen and blood. Separately, blood samples were analysed using an ELISA for detection of antibodies against SQPV (Sainsbury et al., 2000), and skin samples used for detection of SQPV DNA by PCR (following Fiegna 2012).

Spleen samples were collected from 24 adult wood mice (*Apodemus sylvaticus*) trapped using standard little nipper™ snap traps and euthanized inside the red squirrel enclosures between 29/06/2011 and 06/07/2011. Adenovirus DNA was amplified using PCR techniques (as described above for squirrels).

Results

A review of unpublished literature and archived pathological and histological data

SQPV in red and grey squirrels at Welsh Mountain Zoo 1996-8

Prior to the first red squirrels being released at the Welsh Mountain Zoo, archived data revealed that 82% (9/11) of grey squirrel bloods sampled in 1996 were ELISA positive for SQPV antibodies. Following the first red squirrel re-introductions into the woodland on 07/12/1996, when two male and one female were released (see below), a total of nine grey squirrels were sampled between January to April 1997 and all were found to be sero-positive (Tab. 1). These sampled animals were a sub-

Table 1 – Enzyme-Linked Immunosorbent Assay (ELISA) OD_{450} results from blood samples obtained from wild grey squirrels culled in the grounds of the Welsh Mountain Zoo, Colwyn Bay. OD_{450} > 0.2 was taken as the cut off for a positive result.

Sampling period	Sample size	% Seroprevalence	Notes on sample period
Mar 1996 - Sep 1996	11	82%	Sampled prior to red squirrel release Dec 1996
Jan 1997 - Apr 1997	9	100%	Sampled after adult male red squirrel found with SQPV infection, and dead female confirmed with pathological infection in Jan 1997
Mar 1998 - Apr 1998	3	33%	Sampled after a second release of red squirrels

sample from a larger cumulative total of 54 grey squirrels that were shot in the Zoo grounds during the period 02/09/1996 to 18/07/1997. These data demonstrate a continued presence of wild grey squirrels with a history of viral exposure before and during the first re-introduction trial.

A second release of red squirrels was undertaken and one of three grey squirrels killed and sampled in April 1998 was sero-positive; a finding that demonstrated that grey squirrels with antibodies were consistently present within woodland throughout the sampling period during which the two red squirrel release studies took place.

The first trial red squirrel release from Zoo enclosures was on 07/12/1996, and consisted of three animals. One male was last seen on 16/12/1996, whilst a female was found dead in late January 1997 with infection that was confirmed (by TEM tests) as SQPV. The remaining male (Micro-chip BDDO), was found at that time with symptoms typical of SQPV infection; viral particles were subsequently observed under TEM in scabs taken from this sick animal. An ELISA test for antibodies against SQPV on blood collected 03/02/1997 revealed an OD450 reading of 1.81 (cut-off for a positive result = OD450 >0.2). The animal was treated with antibiotics and subsequently recovered sufficiently to be quarantined. Serum from this animal was re-tested by ELISA on 02/09/1997 and the OD450 was found to have declined to 0.51.

In July of that year (1997), screening of four captive red squirrels revealed no antibody response, and these animals were subsequently housed with BDDO. BDDO was released into the wild on 12/12/1997. Following recapture in February and August 1998, repeat testing by ELISA revealed a continued pattern of declining antibody detection (Fig. 2.). The single female that had been released along with BDDO and two other males bred in the Zoo grounds, but unfortunately the last record of a free ranging red squirrel is of an animal found dead in November 1998 and the fate of the other animals in the small population is unknown.

In addition to squirrels included in the 1996-1997 re-introductions, the Moredun viral surveillance archive was found to contain data from an adult female red squirrel which escaped from an enclosure on 21st August 2004. It was found to be ELISA sero-positive (OD450: 0.61) when recaptured on 22nd September 2004, but showed no outward sign of pathological infection and no associated disease was ever recorded during subsequent and prolonged (2 years) captivity.

Only red squirrels which were able to venture out into the woodland surrounding the enclosures at the Welsh Mountain Zoo were found with antibodies or disease. Animals held within the mesh enclosure have not shown evidence of exposure to the virus. ELISA tested bloods (n=1, 03/02/1997, n=3 18/07/1997) were negative and since then there has been no clinical evidence of SQPV infection.

SQPV in other captive red squirrel collections

SQPV infection of captive red squirrels released into woodland containing grey squirrels is suspected in Tilgate Nature Centre, Sussex, England (see Stapleford 2003). Historically grey squirrels were observed on the outer mesh of the red squirrel enclosure. Captive animals were allowed to venture from the enclosure into the surrounding oak and broad-leaved woodland via a 7 cm diameter hole cut through the rear enclosure wall. During a two year period over 200 grey squirrels were shot (G. Clarke *pers. comm.*) and of those sampled, 50% (n=8) were found to be ELISA positive for SQPV antibodies. Red squirrels produced at least two litters in the wild but within a two to three week period all except one animal died from suspected SQPV infection (G. Clarke *pers. comm.*). Unfortunately, diagnosis was confirmed solely by Veterinary clinical examination and no material was examined by TEM for virus particles.

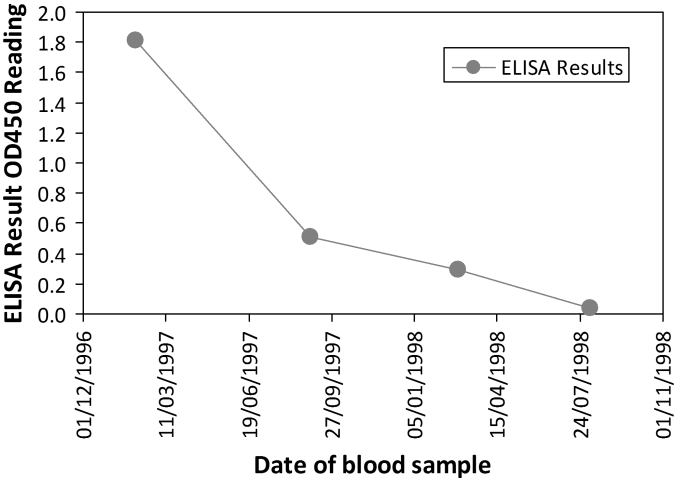

Figure 2 – Enzyme-Linked Immunosorbent Assay (ELISA) OD_{450} Results (Feb 1997 to August 1998) for an adult male red squirrel (PiT identification BDDO) following treatment for TEM confirmed pathological SQPV infection. OD_{450} > 0.2 was taken as the cut off for a positive result. The squirrel was first released into the wild 7/12/1996 and following capture, treatment for squirrel pox and confinement from late January 1997 it was re-released on 12/12/1997.

TEM datum is however available for an adult wild red squirrel trapped under licence in a northern England habitat free from grey squirrels and trans-located to the Alice Holt Research Station in Surrey (SU813427), England in 1995. Storm damage allowed the animal to escape from a woodland enclosure. The adjacent forest contains grey squirrels (Gurnell, 1996) and when re-captured the red squirrel later died from squirrelpox (confirmed by TEM). Similarly, a wild red squirrel caught on the Isle of Wight and released into woodland containing grey squirrels in 1993 (see Kenward and Hodder 1998) was found in our data review (Tab. 2) to have been retrospectively confirmed by TEM about a decade after the event to have contracted SQPV infection.

Cases of SQPV infection in red squirrel collections, confirmed by the presence of virus particles using negative stain TEM, have also been documented in a Cumbria case (2004) (SD103964) and a single case in Staffordshire in 2007 (SK195016). It is known that grey squirrels

Table 2 – Evidence of SQPV infection in captive red squirrel populations or in animals released from captivity. Data obtained from AHVLA and Moredun viral surveillance archives.

Location	Evidence of SQPV infection in captive red squirrels	Evidence of SQPV infection in released captive red squirrels	Notes
Tilgate Nature Centre, Sussex (SU813427)	No clinical pathology and an absence of ELISA data	Clinical pathology but no histological evidence	Grey squirrels active on enclosures and in woodland
Alice Holt, Surrey[1](SU811417)	No ELISA data	Pathological disease confirmed by TEM	Grey squirrels in woodland adjacent to enclosure
Goathorn Peninsula, Dorset[1] (SZ013837)	No ELISA data	Pathological disease confirmed by TEM	Grey squirrels in woodland where red squirrel released
Welsh Mountain Zoo, Colwyn Bay, Conwy (SH836787)	ELISA tests negative	Clinical pathology and disease confirmed by TEM	Grey squirrels active on enclosures and in woodland
Wildwood, Kent (TQ893396)	No ELISA data	No released animals reported	Grey squirrels active on, above and around red squirrel enclosures
Pensthorpe, Norfolk (TF950295)	No ELISA data	No released animals reported	Grey squirrels in woodland adjacent to enclosure
Staffordshire (SK195016)	Clinical pathology and disease confirmed by TEM	No released animals reported	Skin ailments reported in 12 other animals but no mortality

[1] Wild caught animals housed for short period prior to access to wild. In both cases, stock were obtained from habitats without grey squirrels

were active in woodland adjacent to these red squirrel enclosures but unfortunately there is little information relating to activity on enclosures or the circumstances surrounding captive infections. Intriguingly not only are these the only documented cases of red squirrels within enclosures becoming infected by SQPV, but in Staffordshire the clinical pathological case was from within a group of 14 animals housed together. Although two deaths were recorded, only a single body was presented for examination which was SQPV positive by TEM. Of the remaining animals, some were seen to look ill with evidence of scabbing and conjunctivitis and were treated with antimicrobial therapies. No further deaths were recorded and as of two years later, all were appearing healthy.

Additionally, at the Cumbria SQPV site (SD103964) in 2005 and in Cheshire in 2009 (SJ600851) adenovirus cases were reported in mortalities.

Assessing grey squirrel inter-specific viral risk to captive red squirrels at Welsh Mountain Zoo 2011

Grey squirrel activity 2011

All grey squirrel activity on or around the enclosure took place with at least one to three of the four captive red squirrels being active outside of a nest box and active within their enclosure. No obvious direct inter-specific behavioural interactions were observed and the two species appeared to completely ignore each other.

Grey squirrels were observed active on the red squirrel enclosure for a cumulative 47.5 minutes within the 24 hours of observation. Animals were observed grooming and feeding (Fig. 3) but it was not possible to see if any ectoparasites or discarded food actually fell into the red squirrel enclosure during these observations.

It proved difficult to monitor grey squirrel arboreal activity in the dense canopy above the enclosures, but two notable observations were made. The first was on 23/06/2011 10:14:15 am and the second on 24/06/2011 from 10:36:00 am; two occasions when an adult grey squirrel was seen to feed on pine seed above the enclosures for 2 min 45 s and 5 min 15 s respectively. During these periods, debris including cone scales and cores fell into the enclosure containing red squirrels. This provided clear evidence of a potential saliva based infection pathway between wild grey and captive red squirrels.

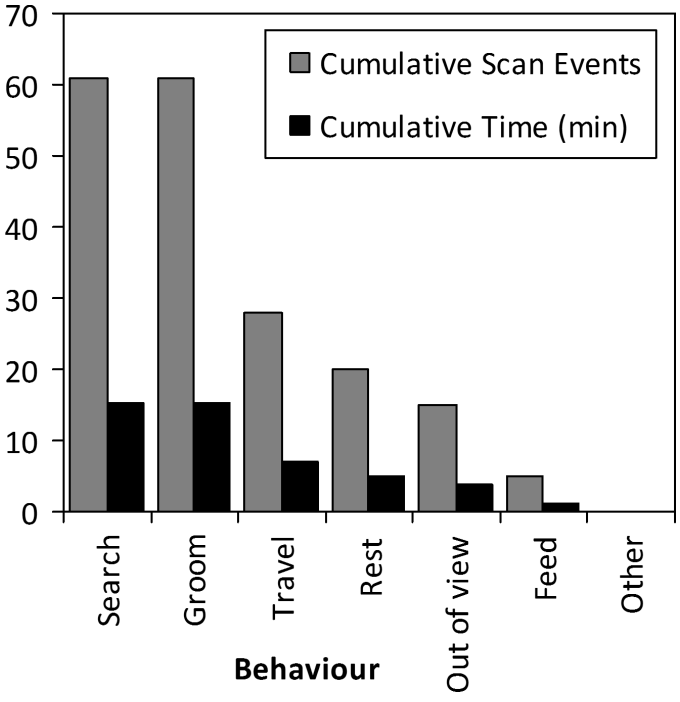

Figure 3 – Grey squirrel activity and behaviour whilst on red squirrel enclosures.

Table 3 – Results of Adenovirus TEM & PCR tests on 18 grey squirrels. PCR results for 24 wood mice are presented for comparative purposes.

	Faeces	Blood	Spleen	Mouse spleen
PCR	3 (17%)	0 (0%)	10 (56%)	2/24 (8%)
TEM	0 (0%)	-	-	-

SQPV 2011

SQPV DNA was not detected by PCR from tissue from any of the 18 grey squirrels examined. In contrast, ELISA blood test results revealed six (33%) of 18 of the sampled individuals had been exposed to the virus at some period in time. Just prior to the 2011 study, a female (PiT tag 11-1589) red squirrel was found dead in the enclosure, but no evidence (either gross pathology or PCR amplification of SQPV DNA) was found that SQPV was the cause of death.

Adenovirus 2011

None of the TEM examined grey squirrels were found to have viral particles in faecal material. Adenovirus DNA was however amplified from faeces and spleen material, but not from blood taken from the same animals (Tab. 3).

Of twenty four mice examined, adenovirus DNA was successfully amplified from 8%. These data demonstrated that adenovirus was present in grey squirrels active on, around, and above the red squirrel enclosure and within wood mice that were active within the enclosure. Unfortunately, the scope of the study did not allow for population estimates to be made for either rodent species and disease surveillance archives do not contain temporal or spatial adenovirus screening for captive populations for which SQPV data are available (Tab. 2).

Discussion

Bruemmer et al. (2010) highlighted the need for greater research into SQPV infection within captive red squirrel collections sympatric with wild grey squirrel populations that are sero-positive for exposure to the virus. Combining otherwise fragmentary information, our retrospective investigation suggests that where captive red squirrels are released (or escape), infection and clinical disease is a strong possibility, and that this occurs across a wide geographic range; a finding that complements earlier published evidence from trans-location studies in Thetford forest, East Anglia (Sainsbury et al., 1997, 2000; Venning et al., 1997). The retrospective index detection of SQPV particles in a red squirrel that was released on Goathorn peninsula in Dorset (Kenward and Hodder, 1998) has also illuminated the involvement of pathogenic disease in this research programme; again re-enforcing the threat which SQPV presents within re-introductions to areas with grey squirrels present.

We also found the earliest temporal cases recorded of a red squirrel surviving squirrelpox and of antibody responses in an animal which was free ranging but did not show signs of disease during the time it was observed. These findings pre-date published accounts (Sainsbury et al., 2008) and perhaps illustrate a need for better co-ordination of information exchange between local conservation initiatives and scientific researchers in relation to emerging red squirrel disease threats. Although it should be stressed that these data do not indicate that red squirrels are becoming immune to SQPV infection, the repeat sampling of antibody levels within a red squirrel exposed to squirrelpox virus in North Wales has provided an invaluable insight into patterns of antibody decline which will help inform future monitoring and interpretation of antibody levels in wild red squirrels elsewhere in the UK.

At the Welsh Mountain Zoo, released and escaped red squirrels encountered SQPV sufficiently for inter-specific infection to occur resulting in pathological disease. In the 2011 study, we were unable to detect SQPV DNA by PCR in the culled grey squirrels and so they may not have been infected at the time of sampling. However, the fact that the grey squirrel population was consistently positive in the SQPV ELISA over a number of years suggests that the virus is circulating in the grey squirrel population. Intuitively, it is likely then that whilst ranging over

a wider forest area, released red squirrels would encounter an active infection in the resident grey squirrels. Although a recent Irish paper (Collins et al., 2014) highlighted urine, ectoparasites and faeces as potential infection pathways, there is no way to retrospectively investigate these potential routes for the captive scenarios we have presented. Nevertheless, from the fact that captive red squirrels are very infrequently found with SQPV we can infer that a single layer of mesh has been sufficient to limit transmission of SQPV to the majority of captive red squirrels collections including Wildwood in Kent and Welsh Mountain Zoo, Conwy where red squirrels have been present with wild grey squirrels for over two decades.

Had both more information and more biological samples been available from Staffordshire, then it may have been possible to identify the most likely inter-specific infection pathway and to understand why twelve other animals within the red squirrel enclosure survived. ELISA and TEM screening would have revealed if intra-specific, or wider inter-specific infection had taken place and we would recommend that such tests are a priority in the future.

Recording the frequency of grooming, feeding and other behaviours by grey squirrels on red squirrel enclosures in Welsh Mountain Zoo, and quantifying periods when stripped pine cone debris dropped directly down into enclosures has, for the first time, quantified potential behavioural routes of inter-specific infection to captive red squirrels in the UK. Where previously it could only be stated that "grey squirrels are active on red squirrel enclosures", this study has illuminated this activity and we would recommend that with advancements in viral surveillance techniques (Real time and nested PCR) that future studies include screening of samples from enclosure mesh, food dropping into enclosures and ectoparasites of grey squirrels resident in the vicinity of red squirrel enclosures.

Greenwood and Sanchez (2002) used an ELISA to detect a high seroprevalence (60%, n=15) of adenovirus in wild grey squirrels at the Welsh Mountain Zoo. In subsequently surveys, although adenovirus surveillance of wild grey squirrels has utilised spleen tissue (Everest et al., 2009) more frequently, it has focussed on blood samples (Everest et al., 2012), often collected as part of annual SQPV monitoring. In this current study, we examined three different sources of material collected from each of the 18 grey squirrels caught and killed at the Zoo (blood, faecal samples and spleen tissue) and found whilst blood was negative, DNA was amplified from 17% of faecal samples and 56% of spleen samples. We would recommend therefore that future monitoring schemes should focus upon tissue analysis, with spleen or intestinal wall preferred tissues of choice.

The 56% infection rate observed in grey squirrels caught within the Zoological Gardens in 2011 parallels the high infection frequency observed previously in captive red squirrels. Everest et al. (2012) found that adenovirus DNA could be amplified from 88% of captive red squirrels found dead at Welsh Mountain Zoo. The authors stated it was difficult to retrospectively differentiate between asymptomatic and clinically-significant infections because of a lack of archived tissue and faecal samples, and despite persuasive histopathology available, no mortality case could be confirmed as more than an association. There remains a possibility that immuno-compromised/stressed individuals may succumb more readily to what would normally be a relatively benign infection in adult animals, but this requires further investigation. It is intriguing that wood mice were also found to be harbouring adenovirus and of course this species is able to move readily in and out of the red squirrel enclosures in a way that sympatric grey squirrels cannot. Whether or not the adenoviruses infecting the wood mice are able to infect squirrels also remains to be determined.

There is an historical paucity of data on adenovirus cases in red squirrels (see Duff et al. 2007) but subsequent surveillance (Everest et al., 2009, 2010, 2012) research, in collaboration with the Zoological Society of Wales, has suggested it as an emerging threat to red squirrel conservation programmes (Everest et al., in press). It is anticipated that increased surveillance for adenovirus will help to clarify its role in squirrel pathologies and whether there is any interaction between this infection and susceptibility to SQPV in grey squirrels.

In conclusion, in the absence of any other studies quantifying red and grey squirrel interactions in the environment, we believe our findings are a helpful addition to the available scientific knowledge. The accumulation of otherwise fragmentary findings will provide useful context for future and more rigorous investigations, into specific inter-specific infection pathways. Finally, our results also underline what the potential disease risk posed by Invasive Alien Species represents to captive breeding programmes, and that re-introductions of native mammals requires careful consideration as recommended in the new IUCN guidelines on re-introductions and conservation trans-locations.

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