Red Squirrels United: Evolving IAS grey squirrel management techniques in the UK

(CSKP0044)

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Front cover image: An Eastern grey squirrel © Paul Harry

Back cover image: A Eurasian red squirrel © Unknown

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Northumberland

Ulster Wildlife

Ymddiriedolaeth Gwiwerod Coch Cymru

Red Squirrels Trust Wales

Newcastle University

3. Summary

Between 2016 and 2020, a project was undertaken, funded by the EULIFE14 (LIFE14 NAT/UK/000467) and National Lottery Heritage Funds in collaboration with the Wildlife Trusts and known as Red Squirrels United (RSU). It was a landscape-scale Eastern grey squirrel (*Sciurus carolinensis*) control programme, undertaken to protect the remaining native Eurasian red squirrel (*Sciurus vulgaris*) population. It involved the removal of large numbers of invasive grey squirrels from four separate areas of the United Kingdom (UK), consisting of nine woodland sites in Lancashire, northern England, Northern Ireland and Wales. Of those removed, a total of 1,506 grey squirrels were necropsied and tissue samples (spleen, lip, hair and whiskers) submitted to the Animal and Plant Health Agency (APHA) laboratory at Weybridge, Surrey, for scientific analysis to determine the presence of amplified DNA from both adenovirus (ADV) and squirrelpox virus (SQPV) in each animal by nested quantitative polymerase chain reaction (qPCR) assays. Of the 1,506 animals submitted for analysis, both ADV and SQPV infection presence was determined from 1,405 of these animals deemed suitable for analysis. Tissue (spleen and lip) and hair (hair or whisker) samples were examined for the presence of amplified ADV or SQPV DNA using these assay platforms.

From the 1,405 animals, 1,378 tissue (spleen and lip) samples were analysed, 43% (598/1,378) positive for ADV and another 10% (136/1,378) positive for SQPV amplified DNA. From 1,031 hair (hair or whisker) samples analysed, 11% (113/1031) were positive for ADV along with another 10% (106/1,031) detected positive for SQPV amplified DNA. Positive animals were detected from all four study areas, with 54% (762/1,405) individual animals detected for either ADV, SQPV or both viruses, from the tissue or hair samples examined. Figures ranged from a lowest detection rate of 47% (40/86) from northern England, to a highest detection of 61% (159/259) from Lancashire. Overall, of 762 animals detected as positive, ADV amplified DNA was detected from 70% (536/762) of them, with a further 15% (111/762) detected positive for SQPV amplified DNA. In addition, a further 15% (115/762) were positive for amplified DNA from both viruses.

4. Introduction

The first identification by transmission electron microscopy (TEM) of squirrelpox virus (SQPV), as the causative agent responsible for squirrelpox disease in the native Eurasian red squirrel (*Sciurus vulgaris*) was made by transmission electron microscopy (TEM) in 1980, in an animal from England, (Scott et al. 1981). Continued detections of the disease have since been made across the remaining red squirrel areas of northern and northwest England. Squirrelpox was first detected in the native sciurid species in Scotland in 2007 from the south of the country (McInnes et al. 2009), from the Tollymore Forest area of Northern Ireland in 2011 (McInnes et al. 2013) and subsequently from mainland Wales in 2017 (Everest et al. 2017a), giving a total geographical spread across the United Kingdom (UK). At the time of writing, several outbreaks across northern England and southern Scotland are being investigated, but the disease is presently absent from both Northern Ireland and Wales.

Adenovirus (ADV) cases in the red squirrel however, are also geographically widespread throughout the UK and are increasingly being reported in the literature. The first cases were also confirmed by TEM at the Veterinary Laboratories Agency at Weybridge in 1997 (Sainsbury et al. 2001*)*, in animals submitted from Suffolk, England. These were from a group of animals, which had been trans-located from Cumbria to boost local populations. Sporadic cases were identified until 2006, when several from Cumbria were confirmed during the year (Duff et al. 2007) and continue to be detected in free-living populations from geographically diverse places as Anglesey (Everest et al. 2012b) and north Wales (Everest et al. 2017b; Shuttleworth et al. 2019b), northern England, Lancashire and Scotland (Everest et al. 2010a, b), Northern Ireland (Everest et al. 2012a) and from captive collections across England and Wales (Everest et al. 2008, 2018).

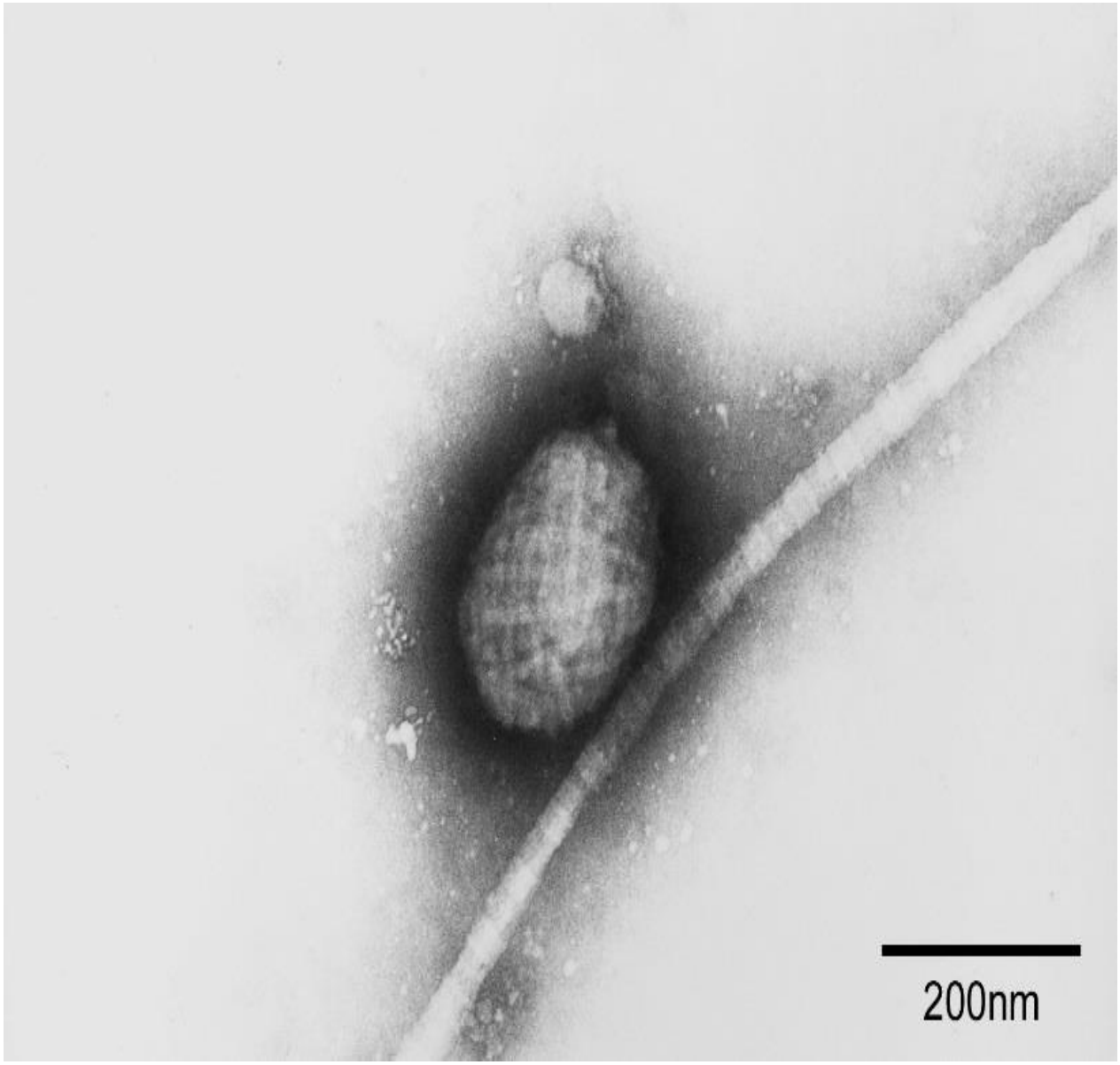
In the UK, both ADV and SQPV are present as asymptomatic infections in the Eastern grey squirrel (*Sciurus carolinensis*), with no pathology observed on examination. Indeed, the species is the immune reservoir for SQPV, where it is essentially unaffected by the virus itself but may facilitate infection transfer to the native species (Rushton et al 2006). One may suspect it acts in the same way for ADV. However, there is but a single report in the literature of squirrelpox disease in the species anywhere in the world, recorded in a UK animal, from England (Figure 1), in 1994 (Duff et al. 1996). To date however, even with all the surveillance undertaken by various groups in the UK, Republic of Ireland and Italy, there have been no pathology recorded due to ADV in the species and no detections of viral particle presence via electron microscopy. For clarity, a micrograph depicting ADV in a red squirrel from England may be seen in Figure 2.

While analyses have shown an ADV presence, both carcass preservation and appropriate analytical sample selection have consistently been an issue and an alternative validated sample source for analysis deemed appropriate. Using material from grey squirrel control programmes across north Wales since 2014 (Cowan et al. 2016), analyses led to the discovery of a regional grey squirrel population exhibiting an asymptomatic ADV presence, through amplified DNA in spleen tissue by polymerase chain reaction (PCR) assays. Archived root bulb material use (whiskers) from these studies provided an opportunity to investigate if a less, or non-invasive sampling process could provide viral presence surveillance studies alongside or in some circumstances, in preference to the more familiar tissue extraction protocols (Everest et al. 2019; Shuttleworth et al. 2019a).

In this study, analyses for both ADV and SQPV virus infection presence were undertaken by nested quantitative PCR (qPCR) assay platforms recently described by Everest et al. (2019), on a range of matched wherever possible, tissue and hair samples from grey squirrels. The samples were obtained from animals on a landscape-scale across the UK, namely from Lancashire, northern England, Northern Ireland and Wales as part of the Red Squirrels United (RSU) project. The animals were trapped and necropsied, with the relevant samples as described, transported to the Animal and Plant Health Agency (APHA) laboratory at Weybridge for analysis and reporting by the PCR assay platforms described.

5. Virus images

Figure 1. An SQPV particle detected by TEM in a grey squirrel from England in 1994, the only known case of squirrelpox disease in the species (Bar = 200nm)



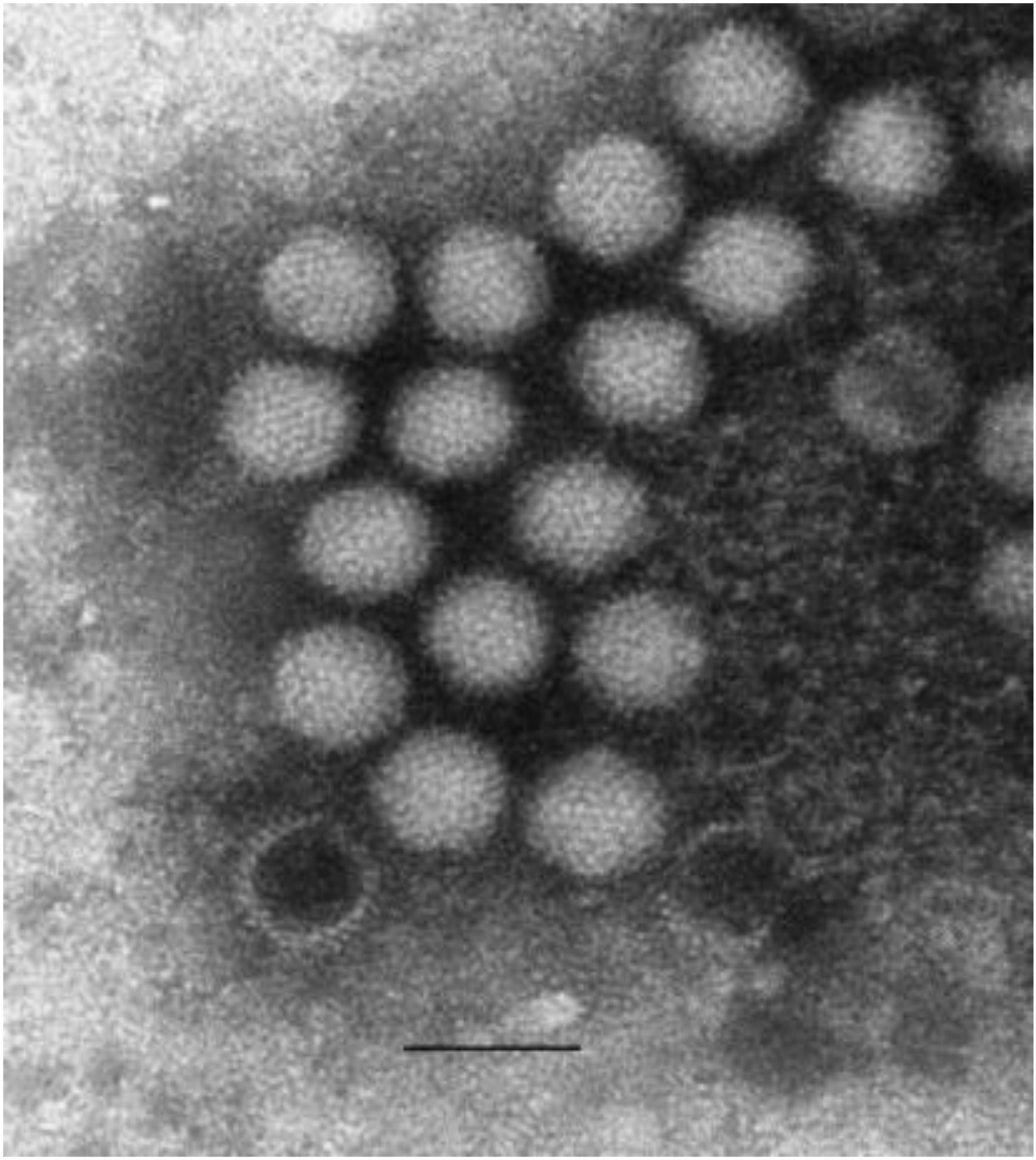
General appearance

Shape: Ovoid

Size: approx. 160nm to 190nm x 250nm to 300nm

Morphology: polypeptide surface spiral wound in a LONGITUDINAL direction

Figure 2. ADV particles detected by TEM in red squirrel intestinal content (Bar = 100nm)



General appearance

Shape: Hexagonal

Size: approx. 70nm to 90nm diameter

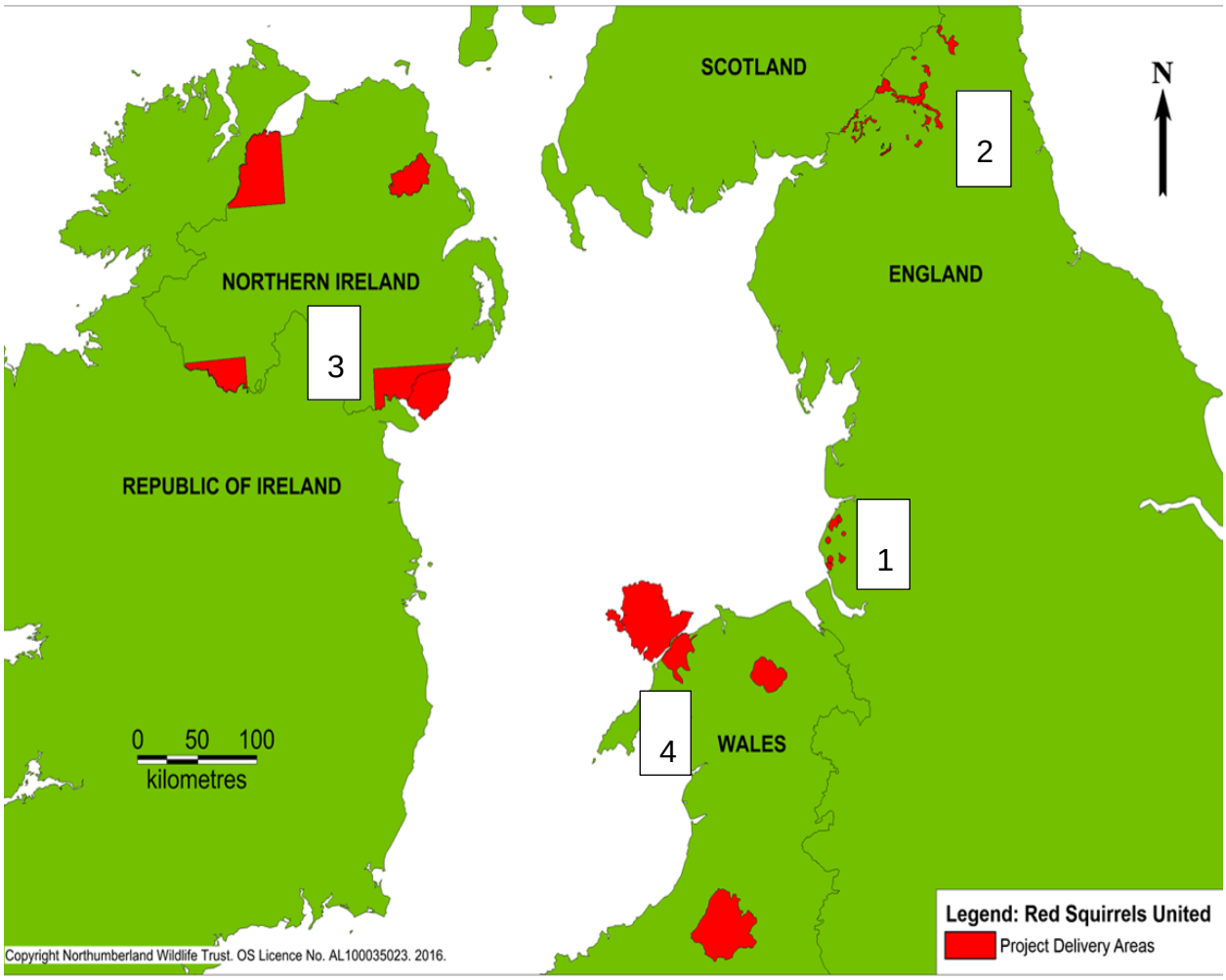
Morphology: Triangular facets on face of particles

6. Materials and Methods

6.1 Materials: Sampling

Between 2016 and 2019, spleen and lip tissue, along with hair and whisker samples were collected for potential analysis by nested qPCR as part of the RSU landscape-scale grey squirrel control study. These animals were representative of the UK grey squirrel population and samples were obtained at post mortem from healthy animals which had been removed from four areas of the UK, comprised of nine woodland sites from Lancashire (LWT), northern England (NWT), Northern Ireland (UW) and north Wales (RSTW). The four study areas may be observed in Figure 3. The samples were received at the APHA laboratory at Weybridge for both ADV and SQPV analysis.

Figure 3. The four RSU UK woodland study areas



Woodland study areas

1 **LWT** (Formby)

2 **NWT** (Kielder complex)

3 **UW** (Mourne mountains)

4 **RSTW** (Gwynedd)

6.2 Materials: Sample preparation

Tissue samples for analysis comprised of a combined spleen and lip sample wherever possible, while hair samples consisted of either 30 hairs/sample, or alternatively, five whiskers. Whole hairs from the tail were used for analysis, while only root bulbs from the whisker were selected. Each sample was placed into an eppendorf vial for extraction and analysis using QIAGEN DNA mini kits (manufacturer's instructions). All sample procedures were undertaken in an MSC Class 1 cabinet. To each sample was added 180ul of ATL extraction buffer and after mixing on a vortex mixer, 20ul of proteinase K (10mg/ml) was also added, mixed and incubated overnight at 56^0C, while agitating at 450rpm. Next morning, following a brief spin to remove drops from the lid, 200ul of AL buffer was added to each sample, mixed and the tubes incubated at 70^0C for 10 minutes and at 450rpm. Again, after a brief spin to remove drops from the lids, 200ul of 100% alcohol was pipetted into each sample tube, mixed and spun at high speed (14,000 x g) to pellet down any material and the whole contents transferred to a clean tube for collection of available DNA by magnetic bead collection using a Kingfisher robotic system prior to nested qPCR analysis.

6.3 Methods: Sample analysis by PCR (magnetic beads extraction)

Use the **NM-LSI_RRC96** protocol on the Kingfisher robotic system.

Add 20ul of well mixed **bead solution** from the LSI MagVet Universal Isolation Kit (Life Technologies) to each well on each labelled sample plate that has 600ul sample present.

Add a **comb plate** to the required position in the robot.

Dispense 100ul of **NM6 Buffer** (elution buffer) into the required number of wells of a shallow well plate and load the plate into the robot.

Dispense 600ul of **80% Ethanol** into the required number of wells of a deep well plate and load this plate into the robot.

Dispense 600ul of **NM4** (wash buffer) into the required number of wells in a 2nd deep well plate and load this plate into the robot.

Dispense 600ul of **NM3** (wash buffer) into the required number of wells in a 3rd deep well plate and load this plate into the robot.

After loading the **sample plate**, start the run. When completed, remove the shallow well plate from the robot and label and seal with adhesive PCR plate sealer to allow the nucleic acid to be safely stored at -20°C until required, or for a few hours in a fridge.

6.4 Methods: Sample analysis by nested qPCR (virus amplification)

All sample and reagent additions were undertaken within an MSC 1 hood.

Components of the Qiagen Quantifast Pathogen PCR kit mastermix are light sensitive so need to be protected from direct or artificial sunlight.

No RNA/DNA should be present in the facility where the mastermix is prepared and pipetted out onto the plate.

Preparation of the mastermix solution/well for both ADV and SQPV assays

Add **7.375ul** of PCR grade water

Add **2.50ul** 5x Quantifast Pathogen PCR mastermix.

Primer set volumes/well (100pmol/ul stock sol.)

Add **0.05ul**	of	ADV HEX-IN-F1	CTC ACT CCT AAC GAA TTC
Add **0.05ul**	of	ADV HEX-IN-R1	CAG TCT TTT GTC ATG TTA C
Add **0.05ul**	of	ADV HEX-OUT-F2	GTG AGT TGG CCT GGC AAT GAT AGA C
Add **0.05ul**	of	ADV HEX-OUT-R2	CGC GGT ACC ATA GCT GCT AAA TCC
Add **0.025ul**	of	ADV HEX PROBE	FAM-CAC ATT GTA TCC TTC TCC ATC G-BHQ1

Add **0.05ul**	of	SQPV POL-IN-F2	GTC AGC ATC AGG TAC ATG
Add **0.05ul**	of	SQPV POL-IN-R2	ACA CCT ACT TTT CCA ACA
Add **0.05ul**	of	SQPV POL-OUT-F2	GAG CGC GAC ACC AGC GAG TTC AG
Add **0.05ul**	of	SQPV POL-OUT-R1	GAG CGT CTC CAA CTC CGC CTT CCT
Add **0.025ul**	of	SQPV_POL PROBE	FAM-ACC GAG GTC CAC GAG ATC AG-BHQ1

Add 10ul mix to each plate well

Samples should be added to the plate mix in a separate room facility.

Add 2.5ul of sample nucleic acid to each well, recording on a form where each sample is situated.

After sample addition, seal the plate and place the plate into a **real time PCR machine** for amplification using the conditions specified below for each assay.

Segment 1 (1 cycle)	95^{o}C for 10 mins
Segment 2 (40 cycles)	95^{o}C for 15 secs
	57^{o}C for 30 secs
	72^{o}C for 30 secs

7. Results

7.1 Analyses

7.2 Grey squirrels

From the many grey squirrels trapped and removed from the four study areas, material from 1,506 animals was submitted to APHA-Weybridge for analysis as being representative of the overall population. Tissue (spleen and lip) and hair (hair or whisker) from 1,405 of the animals was selected and analysed by the nested qPCR assays for ADV and SQPV amplified DNA. A breakdown of all the animals received from each of the four study areas and the corresponding numbers of animals and sample types collected for analysis from the four areas is described in Table 1.

Table 1. Number of animals received, sampled and analysed from each of the four study areas

Area	No. animals received	No. animals sampled	No. of tissue samples analysed	No. of hair samples analysed
LWT	260	259	257	255
NWT	89	86	86	59
UW	486	473	472	276
RSTW	671	587	563	441
Overall	**1,506**	**1,405**	**1,378**	**1,031**

Of 1,378 tissue and 1,031 hair samples selected for analysis (Table 1), 43% (598/1,378) of tissue samples were positive for ADV amplified DNA, ranging from 23% (20/86) from the NWT area, to 48% (122/257) from the LWT area. A further 10% (136/1,378) were positive for SQPV amplified DNA, ranging from 6% (33/563) from the RSTW area, to 29% (25/86) from the NWT area respectively. Of 1,031 hair samples selected for analysis, 11% (113/1,031) were positive for ADV amplified DNA, ranging from 0% (0/59) from the NWT area, to 17% (42/255) from the LWT area. A further 10% (106/1,031) of samples were positive for SQPV amplified DNA, with a low of 5% (13/276) recorded from the UW area, to a high of 13% (58/441) detected from the RSTW area. In addition, 5% (66/1,378) of the previously described positive tissue samples and 2% (15/1,031) of the hair samples analysed, contained amplified DNA from both ADV and SQPV. These results may be seen in Tables 2 and 3.

Table 2. Tissue ADV and SQPV results by nested qPCR from each of the four study areas

Area	ADV	SQPV	Both viruses
LWT	48% (122/257)	11% (28/257)	6% (16/257)
NWT	23% (20/86)	29% (25/86)	6% (5/86)
UW	46% (216/472)	11% (50/472)	5% (22/472)
RSTW	43% (240/563)	6% (33/563)	4% (23/563)
Overall	**43% (598/1,378)**	**10% (136/1,378)**	**5% (66/1,378)**

Table 3. Hair ADV and SQPV results by nested qPCR from each of the four study areas

Area	ADV	SQPV	Both viruses
LWT	17% (42/255)	12% (30/255)	3% (8/255)
NWT	0% (0/59)	9% (5/59)	0% (0/59)
UW	14% (39/276)	5% (13/276)	0.4% (1/276)
RSTW	7% (32/441)	13% (58/441)	1% (6/441)
Overall	**11% (113/1,031)**	**10% (106/1,031)**	**2% (15/1,031)**

From this individual animal data, the percentage figures for the total number of positive sample detections from each of the four study areas for each virus were correlated. Of the 2,409 samples analysed in total, there was 29% (711/2,409) of the samples detected positive for ADV amplified DNA, along with a further 10% (242/2,409) of samples positive for SQPV amplified DNA. In addition, 3% (81/2,409) of these previously described positive samples contained amplified DNA from both viruses. On an individual area basis, there was a lowest value of 14% (20/145) for ADV amplified DNA detected from the NWT area, through to a highest value of 34% (255/748) detected

from the UW area. Alongside of these figures, an additional lowest value of 8% (63/748) for SQPV amplified DNA was detected from the UW area through to a highest value of 21% (30/145) for the NWT area. Therefore, in total, 40% (953/2,409) of the samples were found to be detected positive for either of ADV or SQPV amplified DNA by the nested qPCR assay platforms. The full sample detection results for each of the four study areas may be seen in Table 4.

Table 4. Total positive sample results for each of the four study areas by nested qPCR

Area	ADV	SQPV	Both viruses
LWT	32% (164/512)	11% (56/512)	5% 24/512)
NWT	14% (20/145)	21% (30/145)	4% (5/145)
UW	34% (255/748)	8% (63/748)	3% (23/748)
RSTW	27% (272/1004)	9% (91/1,004)	3% (29/1,004)
Overall	**29% (711/2,409)**	**10% (242/2,409)**	**3% (81/2,409)**

When total numbers of both ADV and SQPV amplified DNA detections from the individual animal samples are collated, an overall assessment of the number of grey squirrels detected as positive for either of the ADV or SQPV amplified DNA can be calculated. Of 1,405 individual grey squirrels analysed from the 1,506 animals received in total from the four project study areas, an overall figure of 54% (762/1,405) of the grey squirrels were identified as being positive for amplified DNA for either of ADV, SQPV individually, or in some cases, for both viruses. Of these 762 animals, 70% (536/762) of them were detected positive for ADV amplified DNA, with a further 15% (111/762) separately detected positive for SQPV amplified DNA individually. In addition to these, a further 15% (115/762) of the animals were detected with amplified DNA present from both ADV and SQPV. Therefore, in total, 85% (651/762) of the animals were detected positive for ADV amplified DNA presence along with 30% (226/762) of the animals as positive for SQPV amplified DNA respectively. All these results are observed in Table 5.

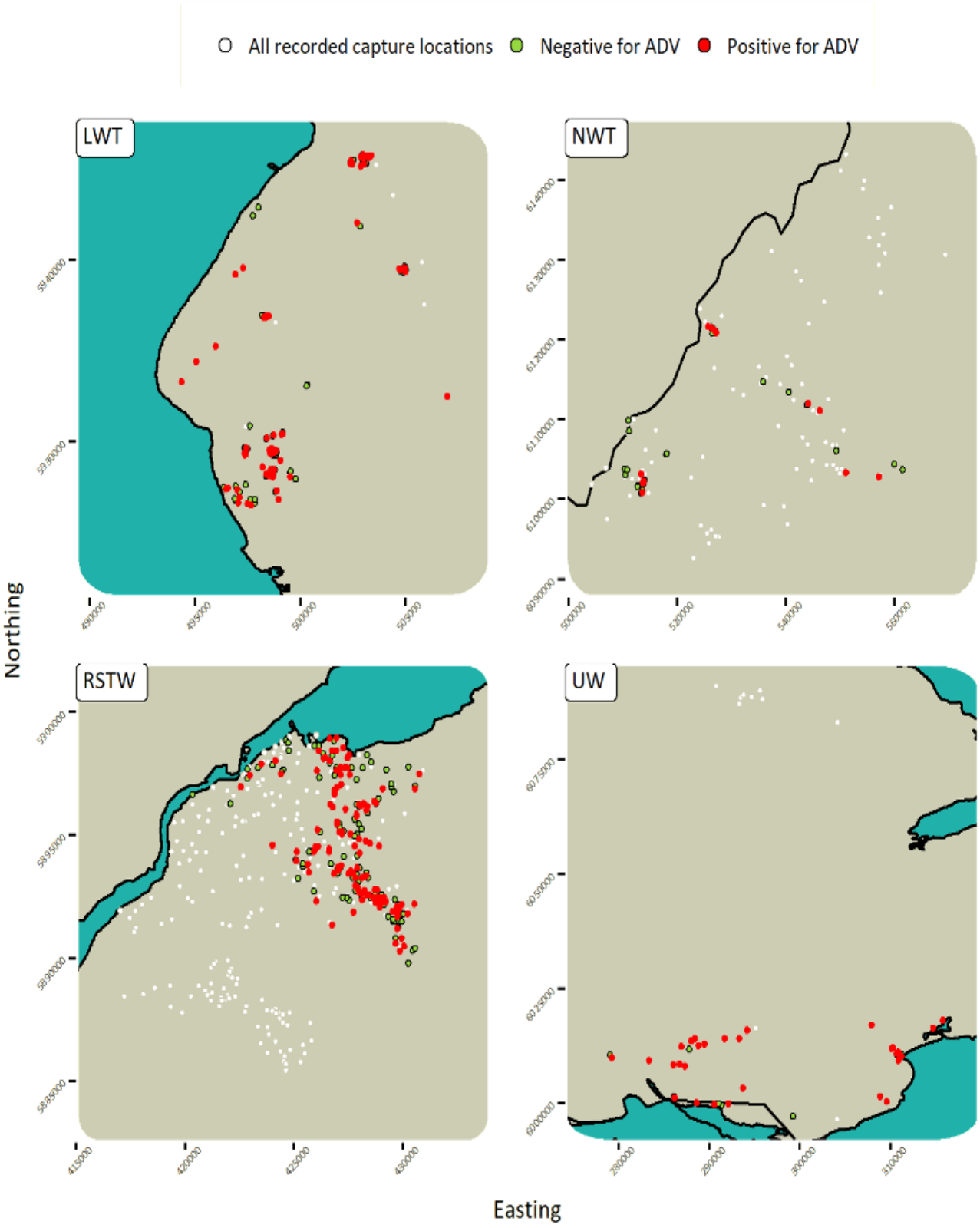
Table 5. Total number of positive animals detected with ADV and/or SQPV amplified DNA

Area	ADV	SQPV	Both viruses	Total
LWT	42% (108/259)	8% (20/259)	12% (31/259)	61% (159/259)
NWT	16% (14/86)	23% (20/86)	7% (6/86)	47% (40/86)
UW	43% (204/473)	6% 30/473)	7% (32/473)	56% (266/473)
RSTW	36% (210/587)	7% (41/587)	8% (46/587)	51% (297/587)
Overall	**70% (536/762)**	**15% (111/762)**	**15% (115/762)**	**54% (762/1,405)**

7.3 Trap location sites

All the available recorded grey squirrel trap capture site locations are listed in the two map sets labelled Figures 4 and 5, being denoted as white circles. All the known locations for positive ADV amplified DNA individual grey squirrel animal detections are denoted as red circles for the positives, while the known locations for the negatives are illustrated as individual green circles on each of the four study areas listed as LWT, NWT, UW and RSTW. These may all be seen in Figure 4. In contrast, all the available known locations for the individual grey squirrel SQPV amplified DNA positive animals are denoted as black circles and the known locations for the negative animals denoted as individual yellow circles on each of the four LWT, NWT, UW and RSTW study areas. These may be seen in Figure 5.

Figure 4. Available trap location sites and known positive and negative ADV amplified DNA detection sites for individual grey squirrels from each of the four study areas



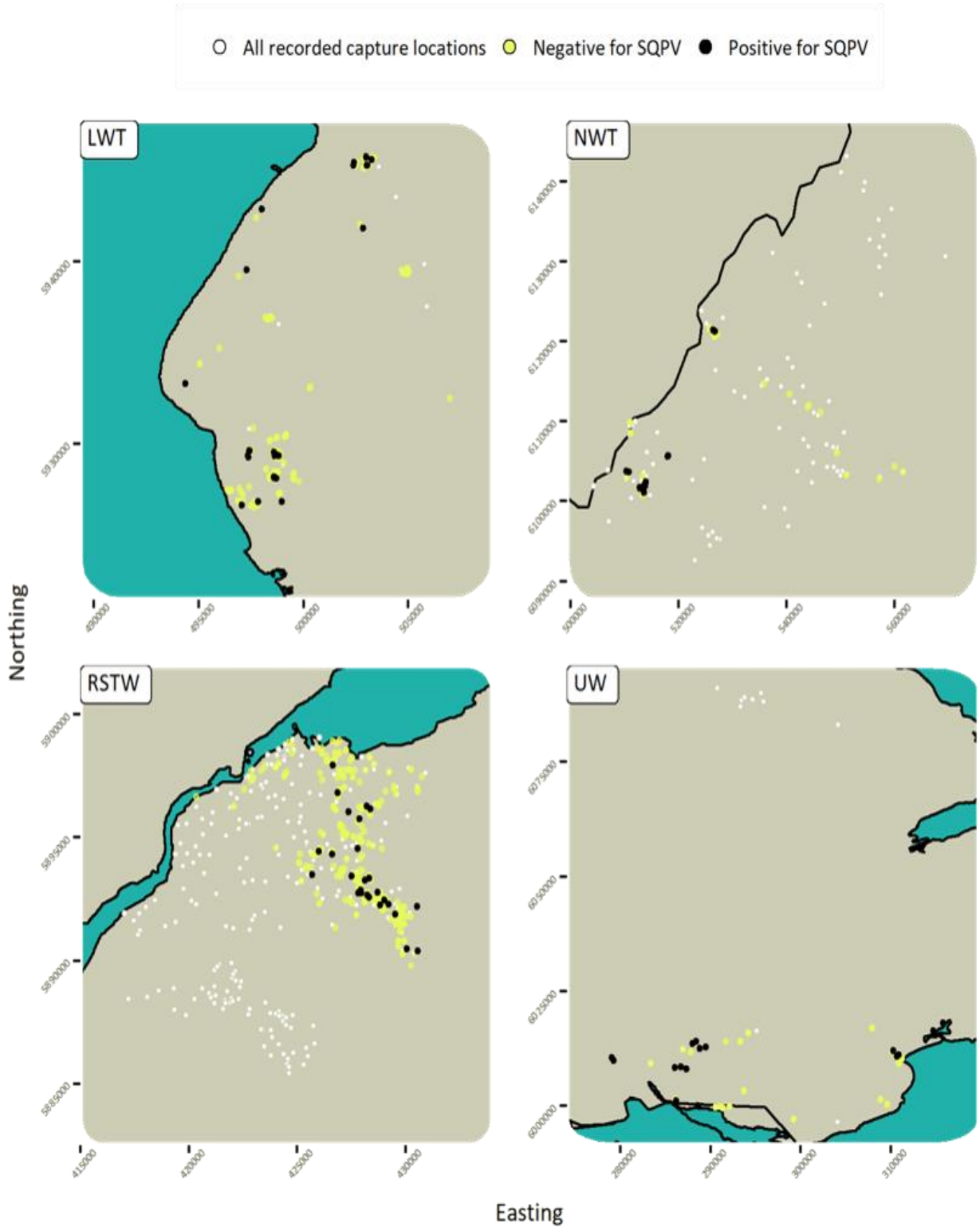
1 **LWT** (Formby)

2 **NWT** (Kielder complex)

3 **UW** (Mourne mountains)

4 **RSTW** (Gwynedd)

Figure 5. Available trap location sites and known positive and negative SQPV amplified DNA detections for individual grey squirrels from each of the four study areas



1 **LWT** (Formby)

2 **NWT** (Kielder complex)

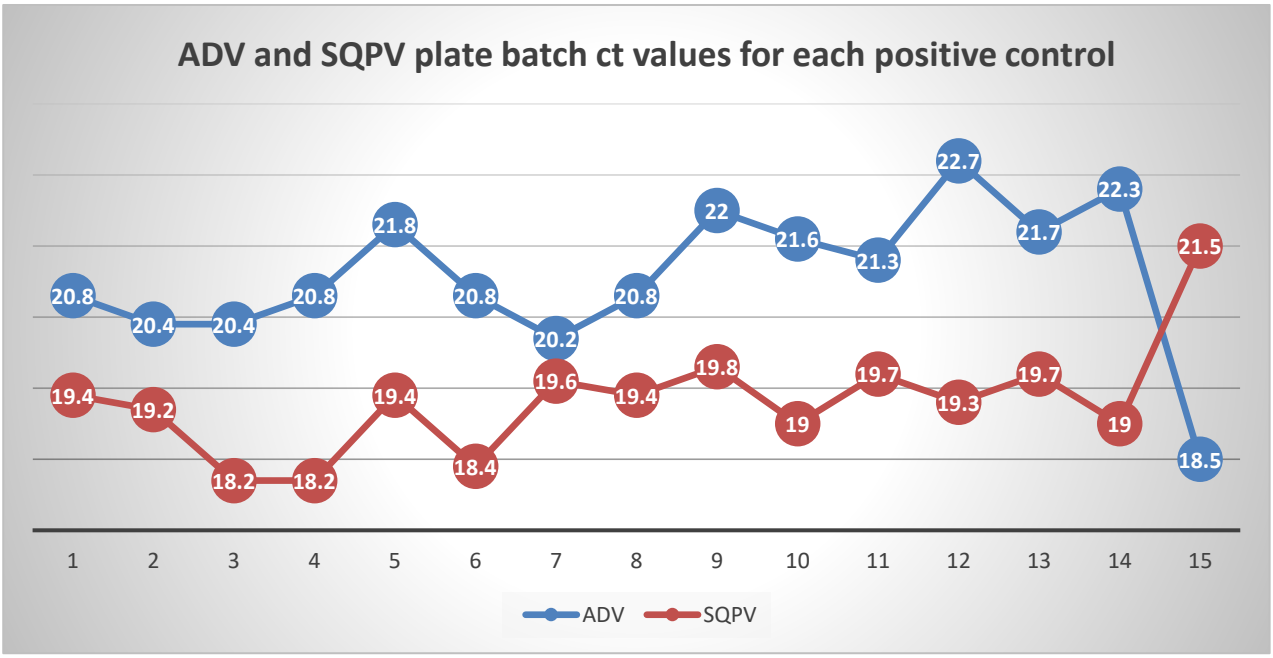
3 **UW** (Mourne mountains)

4 **RSTW** (Gwynedd)

7.4 QC graphs for ADV and SQPV positive control results for the nested qPCR assay platforms

The numbers of individual sample types detected positive for either of ADV or SQPV amplified DNA are described above, as are the numbers or tissues positive from each of the four study areas. The number of individual animals detected positive for either ADV, SQPV or for amplified DNA from both viruses from each study area and in entirety are described in Tables 1 to 5. To ensure that each nested qPCR is passed as correct and fit for purpose, a positive quality control (QC) sample for both ADV and SQPV was run alongside a negative control sample for each sample type (tissue and hair). The negative controls were obtained from a batch of samples which had not been seen to amplify DNA from either ADV or SQPV on any occasion that they had been tested. The positive controls were obtained from a pool of samples analysed by TEM, which had been detected as positive through either ADV or SQPV virus particle presence in either intestinal content for ADV or skin lesion material for SQPV from dead red squirrels. Each positive sample was tested individually to obtain a ct value and then a sub-sample of DNA from each of the selected positive samples was pooled together and then diluted across a range of 1 x 10^2 though to 1 x 10^7. A dilution (1 x 10^6 for ADV and 1 x 10^5 for SQPV) was selected for use with the nested qPCR assay platforms and a pool of material enough for each assay required was prepared. The results of these QC samples in use for each assay are presented as mean assay ct values for each pair of sample plates run over the study period. A mean figure was used, with a +/-3SD ct value incorporated to ensure validity of PCR assays. These results may be seen in Figure 6.

Figure 6. Quality control data for both the ADV and SQPV positive control samples used in both nested qPCR assay platforms and given as amplified DNA ct values



ADV Mean = 21.1, 1sd = +/- 1.30, 3sd = +/- 3.80, Range = 17.30 to 24.90

SQPV Mean = 19.2, 1sd = +/- 0.90, 3sd = +/- 2.70, Range = 16.50 to 21.90

8. Discussion

The RSU project was a large and detailed undertaking, being the first nationwide, landscape-scale grey squirrel control project, with allied scientific surveillance elements to determine the level of both ADV and SQPV asymptomatic infection presence amongst individual grey squirrels in each study area. The project's size, in part, plus being funded by two separate bodies with differing funding structures caused organisational delays to the analytical programme. Incorporating non-governmental organisations with a government (Defra) executive agency such as APHA also caused delays while contracted elements were finalised. Funding structures for each partner was also an issue, while various staffing changes also impacted on sample selection and collection times. The result was that some areas were collecting samples well in advance of others, which ultimately was observed when numbers of samples were eventually received at APHA for analysis.

The project specification was for 300 individual animal spleen and lip samples to be collected from each area for ADV and SQPV analysis respectively. This would give a total of 1,200 animals, with 2,400 samples (1,200 for each virus) and a total of 4,800 PCR analyses. However, with sample collections underway since 2016, part-way through the process in order to take advantage of a newly published non-invasive assay platform using hair and a more sensitive (nested qPCR) assay (Everest et al. 2019; Shuttleworth et al. 2019b), a decision was made to accommodate hair or whiskers as a sample matrix for analysis. This resulted in the need to combine the spleen and lip into a single sample for extraction and analysis, in order to be able to examine matched hair samples wherever possible within the terms of the contract. This had the unintended result that some areas had collected a large part of their samples prior to the decision and had collected fewer hair samples than expected, resulting in a shortfall in the number of samples available for analysis. This is seen in Table 1 for samples received in variance to the original 300 sample figure expected from each study area. Once the samples had been received and catalogued in an APHA sample database, all were given unique consecutive numbers, to allow for easier analysis recording and results reporting, it was apparent there were additional issues to contend with. Firstly, the number of animals available to be sampled and ultimately analysed was reduced through several factors, ranging from no samples present for ones set up in the database, duplicate samples from an animal, through to labels falling off sample tubes and writing on vials rubbed off in transit. In each case, this precluded each of these animal's samples from being analysed. This further distorted the sampling plan, so a decision was taken with full agreement of the programme manager and partners, to analyse additional samples from the areas that collected additional samples, namely UW and RSTW and to match animal tissue and hair samples where possible to rectify any shortfall. In this way, 1,405 animals were tested, from 1,506 received, to ensure a minimum of 1,200 animals were sampled, giving 2,400 samples to achieve the 4,800 corresponding PCR results, 2,400 each for ADV and SQPV.

Amplified DNA spleen positive results obtained for this study identified the grey squirrel as a significant asymptomatic ADV reservoir which may clearly threaten the developing mainland north Wales red squirrel population. This virus has previously been detected in the red squirrel both as an asymptomatic infection, but more importantly, as an agent with a pathogenic impact on the species (Everest et al. 2012b; Shuttleworth et al. 2015), both as a sporadic occurrence in free-living

wild populations, but also in captive red squirrel collections (Everest et al. 2018; Shuttleworth et al. 2019b). A previous study looking at grey squirrels from the same area of north Wales (Cowan et al. 2016) again showed a widespread ADV presence amongst the regional population, with an average detected figure of 58% of animals (186/319) for amplified DNA. The ADV level detected in Wales for this study was only 43% (240/563) from the same area, but an overall study value of 43% (598/1,378) of animals was detected for the four study areas. This may seem at first hand as a lower value, but this study utilised a more sensitive assay platform, along with many more animals (1,378 spleen samples compared to 319 previously). In addition, a previous study, again based on north Wales, focusing on SQPV (Schuchert et al. 2014), reported that with continual control measures, not only could the number of animals with a virus be reduced, but also the extent to which the percentage of SQPV antibodies present could be reduced, so lowering the potential for likely SQPV infection among sympatric red squirrels.

The RSU study examined both ADV and SQPV amplified DNA presence and therefore, likely infectivity, rather than antibody presence and thus previous exposure, but indications of the same scenario are evident. In addition, hair or whiskers were also examined in both studies, with 21% (39/185) positive for ADV in the Cowan et al. (2016) study, compared to the RSU study where 7% (32/441) of samples from Wales were positive, with 11% (113/1,031) overall. For lip SQPV figures, this study produced 6% (33/563) from Wales and 10% (136/1,378) overall. For hair, SQPV gave a 13% (58/441) positive rate for Wales and 10% (106/1,031) overall. The lower ADV figures described for this study may tentatively indicate, even with more sensitive assays, the downward positive detection allied to continued control is an ongoing process. The other three RSU study areas only have limited reported data, but a study by Collins et al. (2014) presented an 8% SQPV amplified DNA figure for squirrel tissue samples, while both Collins et al. (2014) and Shuttleworth et al. (2015) report levels of around 1% SQPV positive detections for hair from Northern Ireland and Wales respectively, correlating with the detection described by Everest et al. (2019). However, another study (Dale and Chantrey 2015) using observations from Lancashire, present tissue SQPV amplified DNA figures of 9%. A further hair study (Chantrey et al. 2014), also based on data from Lancashire, gives an overall detection of 27% for SPQV amplified DNA, corresponding favourably to the Everest et al. (2019) study, which they reported a level of 21% for SQPV amplified DNA.

The rationale for hair in addition to tissue analyses from corresponding animals was to detect ADV or SQPV amplified DNA from each matrix. This would provide evidence of potential environmental presence on the hair, in addition to a tissue presence for the same virus, indicating an active virus infection presence. This would allow for population studies to determine virus infection presence in an area at a given time in grey squirrels. This would also provide an assessment of the likelihood of any sympatric red squirrels present contacting these grey squirrels, or any secretions or tissue material they may have left, which might be absorbed into their bodies via faeco-oral ingestion.

Accumulating virus data using a non-invasive platform such as described by Everest et al. (2019) is now a realistic option, due to ease of collection, such as by remote hair collection via hair tubes, providing primer and probe sequences are available. The viruses in this study were DNA agents, but there are others, some being RNA viruses with zoonotic potential to investigate. These will need to be treated separately to remove DNA present in the sample, so extracted nucleic acid

would need to be split, one half treated with Trizol to obtain RNA for analysis, the other untreated half, used for further DNA virus analysis. In this way, population study dynamics or individual animals may be investigated, reducing overall disease surveillance costs.

This study determined that grey squirrel ADV and SQPV asymptomatic presence across all four study areas is an established feature of each population. For SQPV, several studies (Everest et al. 2009, 2019; Schuchert et al. 2014; Cowan et al. 2016) describe this as a likely reality, with a similar picture being presented for ADV in sympatric free-living red squirrel populations in the UK, with several reports describing such findings (Everest et al. 2010a and b, 2012a and b, 2014; Martínez-Jiménez et al. 2011). In addition, a comprehensive study (Everest et al. 2018) further reported on the considerable ADV presence within captive red squirrel collections across England and Wales.

Our study findings allied with the described viral detection reports in both sciurid species, suggest other inter-species disease transmission pathways could be considered for species other than squirrels (Shuttleworth et al. 2019a) deemed suitable for trans-location and re-introduction. These include mustelid species such as the pine marten (*Martes martes*) where free-living animals from Scotland have been trapped under licence and trans-located to mid-Wales and the west of England. In addition, a small number of captive-bred animals are due for release into north Wales. This carnivorous species preys on small woodland rodent species, such as wood mice (*Apodemus sylvaticus*) and field and bank voles (*Microtus agrestis and Myodes glareolus*) and potentially both sciurid species, each of whom carry an ADV as a clinical manifestation or asymptomatic presence along with other viral agents, such as cowpox, a zoonotic agent. Ongoing grey squirrel control will therefore be a necessary part of a unified approach, until such times as non-lethal control in the form of an immunocontraceptive strategy allied to a red squirrel SQPV vaccine become a reality.

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Never give up on something
that you can't go a day
without thinking about

Winston Churchill