Squirrelpox virus in Northern Ireland: quantifying the risk to red squirrels

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Squirrelpox virus in Northern Ireland: quantifying the risk to red squirrels

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EXECUTIVE SUMMARY

1. Red squirrels (*Sciurus vulgaris*) have been in decline in the UK and Ireland for the last century. This is presumed to be a result of habitat loss and the introduction of the North American grey squirrel (*Sciurus carolinensis*), which competitively excludes the red squirrel and also acts as a reservoir for squirrelpox virus (SQPV) which is almost invariably fatal to the native species.

2. We collated records of red and grey squirrels throughout Northern Ireland (*n*=293) to assess the current (2009-2012) distribution. We also collected grey squirrel tissue samples (*n*=208) from specimens obtained throughout their range in NI to assess the distribution of SQPV. Red squirrel tissue samples (*n*=40) were collected opportunistically.

3. Enzyme-linked immunosorbent assay (ELISA) and quantitative real-time polymerase chain reaction (qPCR) analyses were used to establish the presence of seropositivity (i.e. antibodies) and viral DNA in each specimen sampled. We also examined virus degradation rates in the laboratory under various conditions and tested saliva, urine, faeces and ectoparasites for the presence of SQPV to determine their putative roles as vectors of the disease.

4. The distribution of red and grey squirrels as described in the current study was similar to other recent studies suggesting that grey squirrels are now widespread throughout Northern Ireland and red squirrels remain local and isolated.

5. SQPV was present in most grey squirrel populations tested with 8% of individuals actively infected and 22% of individuals possessing antibodies. Viral loads were typically low.

6. One red squirrel tested positive for the disease at Tollymore Forest Park, Co. Down, possessing both antibodies and virus at high viral loads.

7. Grey squirrels at Killynether Wood, Newtownards possessed no antibodies but had a notably high prevalence of virus (25%) suggesting the disease may have recently spread to the area. This is just 8km north-west of Mount Stewart on the Ards Peninsula which has one of the few remaining isolated red squirrel populations. This area was highlighted as a potential interface between the two species which may present significant disease risks.

8. There was a trend for SQPV infection in grey squirrels to vary significantly between the seasons, being more prevalent in spring and summer than winter. Laboratory work suggested the virus survived for longer in the environment during warm dry conditions.

9. Viral DNA was detected in the faeces and saliva from the positive red squirrel from Tollymore Forest but its absence from the faeces and saliva of positive grey squirrels suggested that environmental spread of the virus may be a viable route of transmission in red squirrels only (due to their high viral load and expression of diarrhoea-like symptoms as observed in the infected red squirrel). The detection of viral DNA in the urine of two grey squirrels suggests that this may also be possible transmission route. However, the most likely source of transmission may be ectoparasites, as all fleas and ticks from the pox positive red, and a third of ectoparasites from pox positive greys tested +ve for SQPV.

10. We make 5 recommendations for conservation action and further research including:

    a) Further surveillance of SQPV within red and grey interface areas;
    b) Establishment of buffer zones around remaining red squirrels perceived as vulnerable to infection (for example, Mount Stewart);
    c) A focal study of a number of mixed populations to determine viral dynamics;
    d) A focal study of seronegative populations harbouring virus;
    e) A focal study of vulnerable red squirrel populations.

CONTENTS

INTRODUCTION

Red squirrels *Sciurus vulgaris* have been in decline in the UK for the last century. This is presumed to be a result of habitat loss and the introduction of the North American eastern grey squirrel *Sciurus caroliniensis* (Gurnell, 1996). Grey squirrels are believed to act as direct competitors to the native red for habit and resources and appear to be able to out-compete the native species (Wauters *et al*., 1999; Gurnell *et al.* 2004; Rushton *et al.* 2006). Additionally, both species are susceptible to the squirrelpox virus (SQPV), a member of the poxviridae family which is relatively similar to the virus that causes Orf in sheep (McInnes *et al.* 2006).

In grey squirrels, SQPV is a sub-clinical infection that rarely manifests in disease (Sainsbury *et al.* 2000; Tompkins *et al.* 2002). However, in red squirrels it causes exudative dermatitis, ulceration with crusted lesions and scabs around the eyes, lips, feet and genitalia which is almost always fatal (Duff *et al*., 1996; Carroll *et al.* 2009). Epidemiological studies using the enzyme-linked immunosorbent assay (ELISA) method for detecting the presence of SQPV-specific antibodies have demonstrated a high presence of SQPV antibodies in healthy greys squirrels, yet an absence of SQPV antibodies in otherwise healthy red squirrels (Sainsbury *et al.* 2000). This indicates strongly that the grey squirrel is a natural reservoir for the SQPV, capable of resisting the disease whilst the red squirrel is not (Sainsbury *et al.* 2008; Carroll *et al.* 2009; Bruemmer *et al.* 2010). Red squirrel populations in close proximity to SQPV seropositive grey squirrels are reported to decline up to 20 times faster than those which reside close to seronegative grey squirrels (Rushton *et al.* 2006). Thus, SQPV is considered a major contributing factor in the decline of red squirrels throughout the UK and seriously threatens current conservation efforts (Rushton *et al*., 2006).

Reports of pox-like infection of red squirrels in the British Isles first appeared in the early 1900s (Middleton, 1930). However, the first confirmed case of SQPV in a red squirrel was in 1981 (Scott *et al.* 1981). Since then there have been multiple outbreaks across the UK. The disease was not known in Ireland until 2011 when two separate incidents were recorded in Northern Ireland; the first at Tollymore Forest Park, County Down during March 2011 and the second at Glenarm, Co. Antrim during June 2011 (McInnes *et al.*, 2012). More recently, there have been two

confirmed cases in the Republic of Ireland; the first at Kilmacanogue, Co. Wicklow, during November 2011 and the second in South Dublin during January 2012 (Sean Callanan, *pers. comms*.). These recent cases of SQPV in red squirrels reflect increasing seropositivity in grey squirrels and emergent cases in red squirrels throughout Ireland (McInnes *et al.* 2012). It is expected, therefore, that as the grey squirrel continues to expand its range and its population size increases, contact rates between the two species will rise and the chances of SQPV spreading to remaining red squirrel populations will also increase (Carey *et al.* 2007).

To date, ELISA has been the only method used in epidemiological studies for the detection of SQPV. However, this represents an incomplete picture of pox occurrence because it tests only for the presence of antibodies (an indicator of past infection) and cannot provide data on current infection rates. Information on both the presence of virus particles (as assessed by detection of viral DNA) and their concentrations in red and grey squirrel populations is necessary to understand the spread of the disease. Quantitative real-time PCR (qPCR) is a highly sensitive and reliable technique used to amplify fragments of DNA. This technique can be used to infer the presence of virus particles in squirrel tissue, thereby providing information on the presence of pox virons and the level at which these particles are present.

SQPV transmission routes from grey to red squirrels and from red to red individuals have not been explored. It has been hypothesised that direct contact between infected animals, shedding of pox virons (or DNA) into the environment and the involvement of ectoparasites as vectors are the primary means of transmission (Atkins *et al.*, 2010; Bruemmer *et al.*, 2010). Developmental work in these areas is required to understand SQPV epidemiology to predict levels of risk to remaining red squirrel populations and to inform future conservation efforts.

The principal objective of this project was to use both ELISA and qPCR to assess past exposure and current infection of grey and red squirrels to SQPV across Northern Ireland. Saliva, urine, faeces and ectoparasites from both species were examined for the presence of pox virons, via DNA amplification, to investigate their potential as a means of disease transmission.

METHODS

Survey of squirrel distribution

The current distribution of red and grey squirrels was assessed by a questionnaire (Appendix 1) distributed to conservationists, local conservation rangers and park officials. The questionnaire asked respondents if sightings had been made in specific forests of reds only, greys only or mixed (an area where red and grey squirrels co-exist) populations and the approximate period over which recent sightings had been noted. These data were combined with records obtained from the Centre for Environmental Data and Recording (CEDaR), Northern Ireland Environment Agency and Northern Ireland Forest Service. Only records from 2009 to 2012 were used and a distribution map created.

Sample collection

Grey squirrels were trapped under Government license (see Acknowledgements) in a range of forests throughout Northern Ireland. Traps of 175 x 150 x 600mm (Albion Manufacturing; ALBI 079 squirrel trap), were baited with peanuts (50%), maize (25%) and sunflower seeds (25%) and set overnight and checked early the next morning and subsequently at 3 hour intervals throughout the day. Captured grey squirrels were euthanized by neck dislocation in accordance with the Northern Ireland Squirrel Forum's (NISF) code of practice. Culled grey squirrels were also supplied by Northern Ireland Forest Service Rangers, members of local Red Squirrel Conservation Groups and members of the public. No red squirrels were culled in this study, but carcasses were collected as a result of natural death, road traffic accidents, disease or predation. All carcasses were frozen at -20°C for storage and defrosted prior to tissue harvesting and processing. Each squirrel was sexed and weighed.

Saliva sampling

Saliva was collected from twelve grey and one red squirrel and extracted from their defrosted carcasses. Samples were collected using a double swab technique to maximise DNA recovery (Sweet *et al*., 1997). Two swabs, the first dipped in sterile water, the second dry, were rolled over each side of the buccal cavity and tongue in a circular fashion with moderate pressure.

Urine sampling

The bladders of 133 grey squirrels were dissected out and examined for the presence of urine. A total of 38 squirrels appeared to have urine present which was extracted by clamping the bladder using arterial forceps and removing it from the carcass using a scalpel. Urine was extracted by piercing the bladder using a 23 gauge 31.75mm needle.

Faecal sampling

A section of the lower intestinal tract was removed from twelve grey squirrels and one red squirrel. Faeces samples from individual squirrels were separated from the intestinal wall using a scalpel to minimise contamination of non-shed intestinal wall.

Identification of potential vectors

Ectoparasites were identified and extracted from 14 grey squirrel and one red squirrel carcass by combing the coat. Each parasite was identified as a flea, tick, or mite. A sample of ectoparasites from six grey squirrels and one red were tested for SQPV.

ELISA analyses

Squirrel blood was extracted from each carcass using a 23 gauge 31.75mm needle and sent to Moredun Research Institute (Midlothian, Scotland) for enzyme-linked immunosorbent assay (ELISA) analysis to test for the presence of SQPV antibodies.

ELISA methodologies were those previously published by McInnes *et al.* (2006). Results were categorised as either positive if corrected OD values were >0.2 or negative if <0.2. The distribution of SQPV antibodies in populations of both grey and red squirrels was mapped using ArcGIS 10.1 (ESRI, California, USA).

DNA extraction

DNA from a 25mg segment of the lower lip from each squirrel and from whole ectoparasite samples was extracted using the DNeasy Blood and Tissue kit (QIAGEN) following the manufacturer's instructions. DNA from saliva and urine samples extracted using the QIAamp DNA Micro Kit (QIAGEN) following manufacturer's instructions. DNA from faecal samples was extracted using TRIzol Reagent (Invitrogen) following manufacturer's instructions. DNA equivalent to 0.25mg of lip tissue, 1µl of blood, 1/10 of total ectoparasite DNA and 1/4 of saliva and urine DNA was added to each PCR reaction. Published forward and reverse SQPV primers described in Atkins *et al.* (2010) were used to detect pox virus DNA. qPCR analysis was performed on the Bio-Rad CFX96 C1000 using Fast start SYBR Green Master (Roche) according to manufacturer's instructions, under the following cycling conditions: (95°C x 10min, 40 x (95°C x 10s, 65°C x 10s, 72°C x 20s) 72°C x10 min (Atkins *et al.* 2010). To ensure primer specificity and that amplicons were of the correct length, products were analysed by melt curve analysis and checked on a 0.4% agarose gel containing 0.0075% ethidium bromide from a 10mg/ml stock.

qPCR analysis

qPCR protocols, including sensitivity and specificity of the reaction, were developed and optimised by Atkin *et al*. (2010). Samples were classed 'positive' if they produced a strong amplification signal that was different from negative and no template controls together with a melt of 87-88°C. On those occasions where negative controls were positive, the sample with an additional negative control was repeated. Quantification of the viral load was determined by the standard curve method using a serial dilution of a SQPV standard (Atkin *et al*., 2010). Virus load in squirrel lip and faeces was calculated using a standard diluted in negative squirrel DNA and negative faeces DNA respectively to account for observed inhibition in

amplification. Virus load in blood, ectoparasites, saliva and urine was calculated using the standard diluted in water as no PCR inhibition was observed. The standard was produced from the reaction of SQPV forward and reverse qPCR primers on a positive squirrel sample, the resulting amplicon was gel extracted, cloned using 'Qiagen PCR cloning kit' following manufacturer's instructions and sequenced using the 'Dundee Sequencing Service.' Data presented on viral load is the inferred number of virus particles present in a milligram of tissue (v/mg), present in one ml of blood (v/ml), a whole ectoparasite specimen (v/e), swabbed saliva (v/s) and one ml of urine (v/ml) deduced from genomic equivalents.

Virus degradation rates

The viability of the virus in the environment was investigated under two conditions (wet or dry) and three temperatures (5°C, 15°C and 25°C) for a period of one month. A section of scab from an infected red squirrel which died of squirrelpox (donated by Prof. Sean Callanan, University College Dublin) was used as a source for the virus. The scab was dried over silica gel for a period 36 hours in a dessicator (Livingston and Hardy, 1960), sectioned into small pieces using a scalpel with equal amounts aliquoted into separate Eppendorfs. Those Eppendorfs which were to be left in 'dry' conditions were sealed in an air tight polythene zipper bag with silica gel crystals placed at the bottom, this bag was in turn sealed in a larger air tight polythene zipper bag. A volume of distilled water roughly equal in volume to the amount of dried scab in each Eppendorf was added to those samples which were to be left in 'wet' conditions. These 'wet' samples were sealed in an air tight polythene zipper bag with a damp cloth at the bottom; this bag was in turn sealed in a larger air tight polythene zipper bag. After a period of 1 month, samples were analysed by 'Negative Contrast Stain Transmission Electron Microscopy' for the presence of identifiable virus particles (Everest *et al*., 2010). The numbers of degraded and intact particles detected were enumerated.

Statistical analyses

The distribution of red and grey squirrel and positive ELISA and qPCR results (from lip and blood combined) were mapped using ArcMap 10 (ESRI, California, USA). Descriptive cross-tabulation was used to assess the sensitivity and specificity of the qPCR and ELISA as techniques for capturing the disease status of red and grey squirrels.

The occurrence of positive results for: a) qPCR; b) ELISA; and c) overall disease status (both species and test results combined) was examined using Generalized Linear Mixed Models (GLMMs) assuming a binomial error structure and a logit link function. 'Forest' was fitted as a random factor to account for multiple squirrels collected from each location. Disease status (infected or uninfected) was fitted as the dependent variable. Variance was examined with respect to locality, species, sex, the occurrence of ectoparasites and season (all fitted as fixed factors).

The numbers of degraded and intact virus particles remaining after one month of exposure to simulated environmental conditions were analysed using Generalized Linear Models (GLMs) assuming a Poisson error structure (for count data) and a logarithmic link function. Temperature category (5, 10 and 15^{o}C) and Condition (wet or dry) were fitted as fixed factors including the two-way interaction.

Power Analysis was conducted to determine the likely sample size of squirrels required to detect changes in the prevalence of SQPV. This assumed a 2x2 χ^2 contingency format to test for changes between the number of positives verses negatives between two survey periods (i.e. the current study and any future study). Three levels of effect size were examined: a small change (10%), a moderate change (30%) and a large change (50%).

RESULTS

Survey of squirrel distribution

A total of 293 records for red and grey squirrels were collated from 2009 to 2012. A total of 186 x 10km squares covered Northern Ireland, with 24 (13%) cells containing red squirrel records only, 26 (14%) cells containing grey squirrel records only, 54 (29%) containing records of both species and 82 (43%) containing no records of any squirrels. Red and grey squirrels and their coincident occurrence was widespread (Fig. 1).

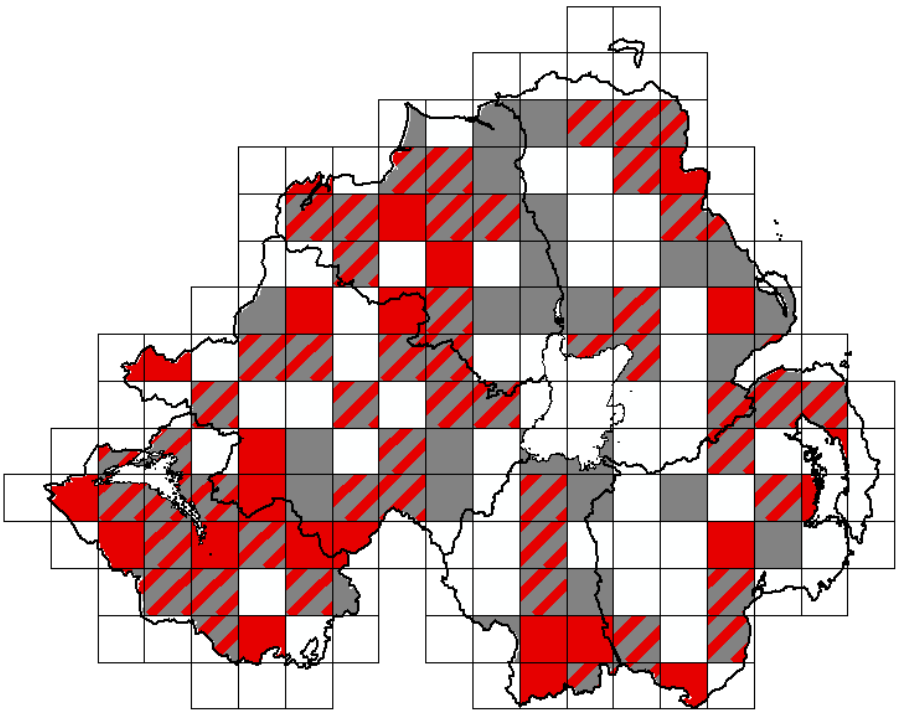

Figure 1. Distribution atlas map (10km scale) showing cells containing records for red squirrels (red), grey squirrels (grey) and both species (hatched) throughout Northern Ireland from 2009-2012.

Sample and tissue collection

Squirrel carcasses were collected from 37 individual forests within 17 discrete localities throughout Northern Ireland from April 2010 to June 2012 (Fig. 2). A total of 208 grey squirrels and 40 red squirrels were collected (Table 1).

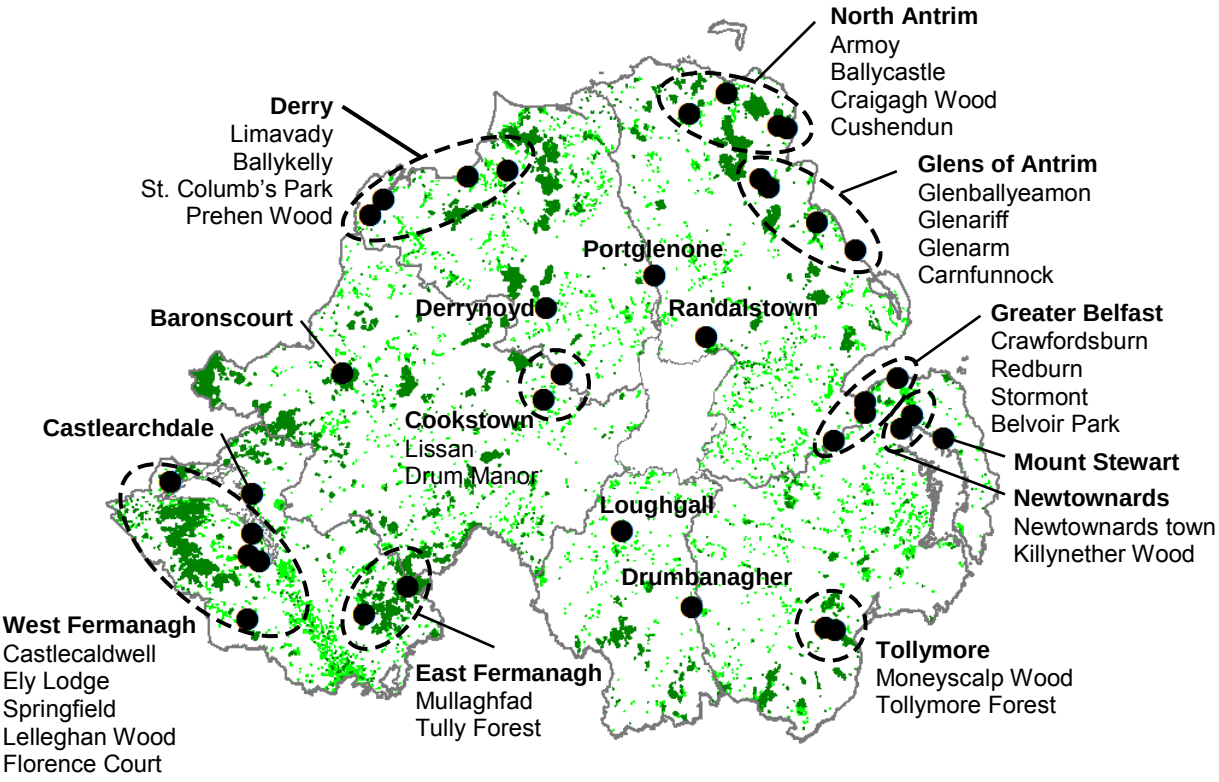

Figure 2. Sampling locations i.e. forests (*n*=37) from which squirrel carcasses were retrieved and their groupings (dashed lines) into localities (bold text) for the purposes of analysis (*n*=17). Light green represents Broad-leaved woodland and dark green represents Coniferous plantations or Mixed forest.

Table 1. The number of grey and red squirrel carcasses collected and their forests of origin.

Locality	Forest	Population status	No. of carcasses		
			Grey squirrels	Red squirrels	Total
1. Baronscourt	1. Baronscourt Estate	Mixed	6	2	8
2. Castlearchdale	2. Castlearchdale Estate	Mixed	4	-	4
3. Cookstown	3. Drummanor	Grey only	10	-	10
	4. Lissan	Grey only	12	-	12
	Sub-total	Grey only	22	-	22
4. Derry	5. Ballykelly	Mixed	10	-	10
	6. Limavady	Red only	-	1	1
	7. Prehen Wood	Red only	-	1	1
	8. St. Columb's Park	Red only	-	1	1
	Sub-total	Mixed	10	3	13
5. Derrynoyd	9. Derrynoyd	Grey only	10	-	10
6. Drumbanagher	10. Drumbanagher	Grey only	20	-	20
7. East Fermanagh	11. Mullaghfad Forest	Red only	-	1	1
	12. Tully Forest	Red only	-	3	3
	Sub-total	Red only	-	4	4
8. Glens of Antrim	13. Carnfunnock	Grey only	2	-	2
	14. Craigagh Wood	Grey only	1	-	1
	15. Glenariff	Red only	-	4	4
	16. Glenarm	Mixed	4	-	4
	17. Glenballyeamon	Red only	-	1	1
	Sub-total	Mixed	7	5	12
9. Greater Belfast	18. Belvoir Park	Mixed	8	-	8
	19. Crawfordsburn	Mixed	6	-	6
	20. Redburn	Mixed	10	-	10
	21. Stormont	Mixed	7	-	7
	Sub-total	Mixed	32	-	32
10. Loughgall	22. Loughgall	Grey only	14	-	14
11. Mount Stewart	23. Mount Stewart	Red only	-	8	8
12. Newtownards	24. Killyneather Wood	Mixed	10	-	10
	25. Newtownards town	Mixed	2	-	2
	Sub-total	Mixed	12	-	12
13. North Antrim	26. Armoy	Red only	-	1	1
	27. Ballycastle	Mixed	20	4	24
	28. Cushendun	Red only	-	2	2
	Sub-total	Mixed	20	7	27
14. Portglenone	29. Portglenone	Grey only	21	-	21
15. Randalstown	30. Randalstown	Grey only	20	-	20
16. Tollymore	31. Moneyscalp Wood	Red only	-	1	1
	32. Tollymore	Mixed	8	5	13
	Sub-total	Mixed	8	6	14
17. West Fermanagh	33. Castlecaldwell Forest	Red only	-	1	1
	34. Ely Lodge	Mixed	-	1	1
	35. Florence Court	Mixed	-	2	2
	36. Lelleghan Wood	Red only	-	1	1
	37. Springfield	Grey only	3	-	3
	Sub-total	Mixed	3	5	8
	Grand Total	Mixed	**208**	**40**	**248**

ELISA and qPCR

A total of 54 (22%) of 248 squirrels tested positive for SQPV antibodies using the ELISA technique compared to 19 (8%) testing positive for virus particle DNA using the qPCR technique on lip tissue and 4 (2%) of 243 squirrels using qPCR on blood (Table 2). The results of ELISA and lip tissue qPCR matched in 74% of cases, ELISA and blood qPCR in 77% of cases, lip qPCR and blood qPCR in 92% of cases and ELISA and the combined results from both lip and blood in 72% of cases. Only 4 squirrels (2%) tested positive for both antibodies and virus particles in either the blood or lips.

Table 2. Descriptive cross-tabulation summary of ELISA and qPCR results. Values represent the numbers of squirrels (of both species combined) with percentages in parentheses.

		Lip qPCR		
		-ve	+ve	**Total**
ELISA	-ve	179 (72%)	15 (6%)	**194 (78%)**
	+ve	50 (20%)	4 (2%)	**54 (22%)**
	Total	**229 (92%)**	**19 (8%)**	**248**

		Blood qPCR		
		-ve	+ve	**Total**
ELISA	-ve	188 (77%)	3 (1%)	**191 (79%)**
	+ve	51 (21%)	1 (<1%)	**52 (21%)**
	Total	**239 (98%)**	**4 (2%)**	**243**

		Lip qPCR		
		-ve	+ve	**Total**
Blood qPCR	-ve	222 (91%)	17 (9%)	**239 (98%)**
	+ve	2 (1%)	2 (1%)	**4 (2%)**
	Total	**224 (92%)**	**19 (8%)**	**243**

		Combined qPCR results		
		-ve	+ve	**Total**
ELISA	-ve	177 (71%)	17 (7%)	**194 (78%)**
	+ve	50 (20%)	4 (2%)	**54 (22%)**
	Total	**227 (92%)**	**21 (8%)**	**248**

A total of 53 (25%) of 208 *grey* squirrels tested positive for antibodies and were widespread in N. Ireland (Fig. 3a), but prevalence within localities varied from 0 - 60% (the latter being Derrynoyd, Co. Londonderry). This compared to 20 (10%) of grey squirrels testing positive for virus particle DNA (either in the lip or blood) which were less widespread than for the antibodies (Fig. 3b) but prevalence within localities varied from 0 - 25% (the latter being Newtownards including Killynether Wood, Co. Down). Only 3 greys (1.5%) tested positive for both antibodies and pox virus DNA. A total of 1 (2.5%) of 40 red squirrels tested positive for both antibodies and virus particle DNA (Fig. 3c-d) but prevalence within localities varied from 0 - 17% (the latter being Tollymore Forest Park). Using the results of all three tests suggested that the disease was widespread throughout Northern Ireland between 2010 and 2012 and present in most localities invaded by grey squirrels (Fig. 3e).

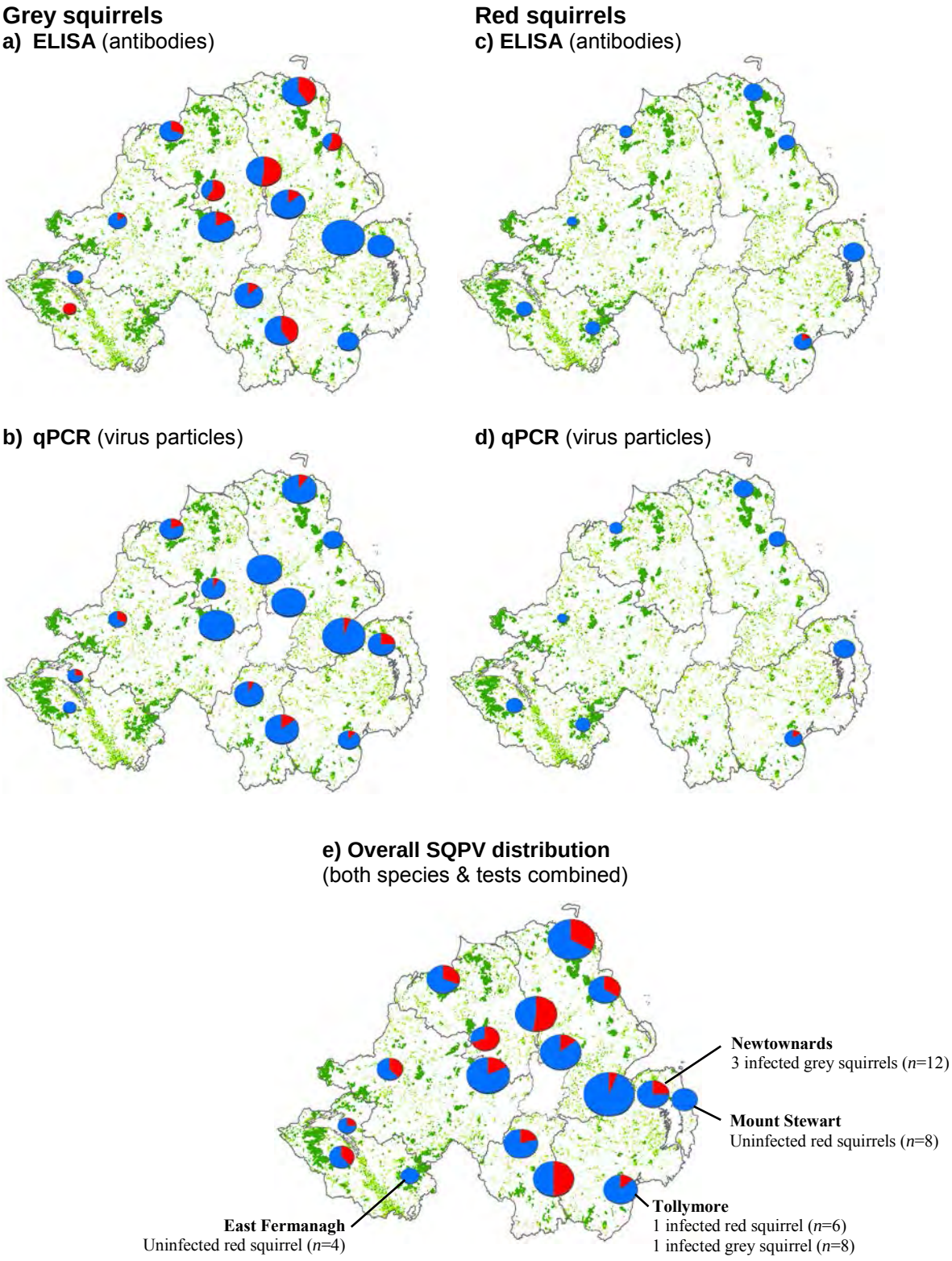

Figure 3. Distribution of squirrelpox virus throughout Northern Ireland from 2010 to 2012 within grey (left) and red squirrels (right) using **a & c)** ELISA and **b & d)** qPCR and **e)** both species and tests combined (i.e. overall distribution of the disease). **Red** = positive, **blue** = negative and the size of the chart is scaled for sample size i.e. number of squirrels tested at each locality.

Grey squirrels were present in most localities from which red squirrels were tested (compare Fig. 1 and Fig. 3e), the exceptions being Mount Stewart, Co. Down and East Fermanagh (i.e. Mullaghfad and Tully Forests). It was notable that grey squirrels in some localities, for example the Greater Belfast and Newtownards areas (including Killynether Wood), presented no antibodies but were infected with virus particle DNA suggesting that the disease may have spread to this area only recently. Moreover, a quarter of all squirrels were infected in the Newtownards area which is only 8km north-west of Mount Stewart, which supports a large red squirrel population, with reds also occurring further south, such as in Greyabbey, Kircubbin and Portaferry (J. Dick *pers. obs*.).

The viral load in lip (representative of the amount of virus genome present) was low for infected grey squirrels (median = 63 v/mg: mean = 395 v/mg). A total of 9 grey squirrels (50%) had viral loads greater than the median and only 1 individual had a load that could be considered moderately high at 4901v/mg (collected at Loughgall Estate, Co. Armagh). In comparison, the viral load of the only positive red squirrel was almost 50,000 times higher than the median viral load for grey squirrels at 3,113,957 v/mg. Only 3 grey squirrels tested positive for squirrelpox virus DNA in the blood (median = 8,370 v/ml: mean = 12,990 v/ml) and the one red from Tollymore 21,032,512 v/ml. The higher levels of virus in the blood is primarily due to the greater amount of tissue used for standardisation; overall there was lower rates of positive response in blood.

There were no correlates of virus particle occurrence as determined by qPCR (Table 3a), however, the presence of antibodies (i.e past exposure) varied significantly between localities whilst antibodies and overall disease status (antibodies plus qPCR) varied significantly between species and season (Table 3b & c). Occurrences of seropositive and DNA positive squirrels were significantly greater in grey than red squirrels (Fig. 4a) and significantly lower in winter than spring and summer (Fig. 4b).

Table3. Generalized Linear Mixed Model (GLMM) results for predicting disease status using **a)** qPCR, **b)** ELISA and **c)** both combined.

Model / Parameter	*F*	*β* ± s.e.	df	*P*
a) Combined qPCR				
Locality	0.42	Factor	14	0.969
Species	0.00	Factor	1	0.999
Sex	0.01	Factor	1	0.910
Ectoparasite presence	0.41	Factor	1	0.521
Season	2.36	Factor	3	0.070•
b) ELISA				
Locality	**2.11**	**Factor**	**12**	**0.019****
Species	**7.84**	**Factor**	**1**	**0.006****
Sex	2.50	Factor	1	0.116
Ectoparasite presence	0.00	Factor	1	0.994
Season	2.46	Factor	3	0.065•
c) Overall disease occurrence				
Locality	1.04	Factor	16	0.414
Species	**12.85**	**Factor**	**1**	**<0.001*****
Sex	0.74	Factor	1	0.389
Ectoparasite presence	0.02	Factor	1	0.877
Season	**3.33**	**Factor**	**3**	**0.019****

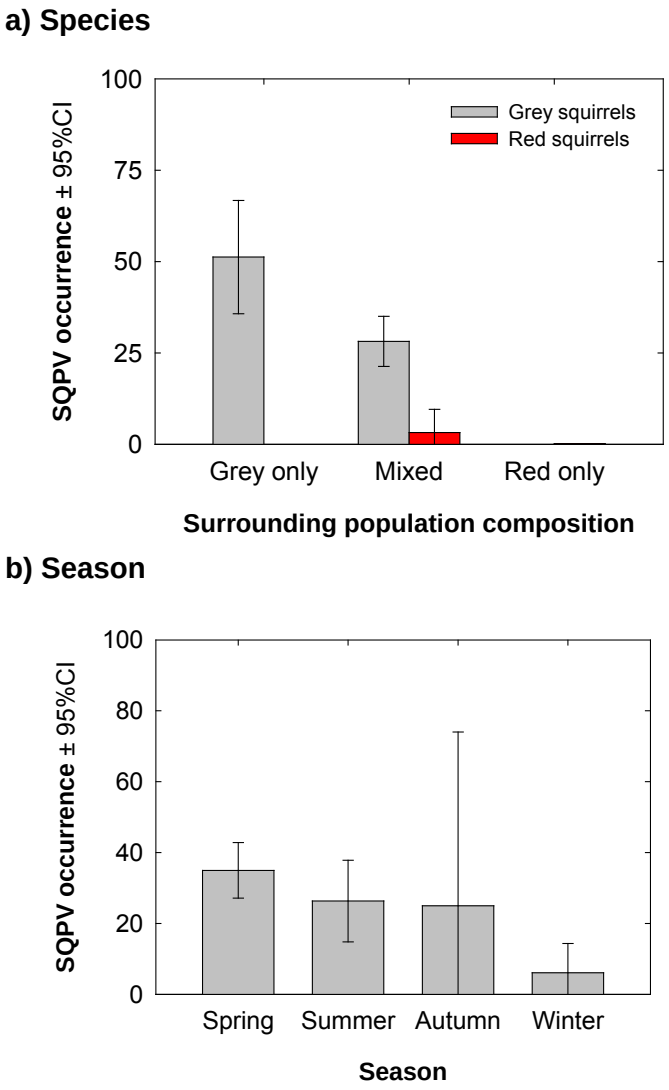

Figure 4. Overall squirrelpox occurrence (percentage of squirrels infected (both species and tests combined) ± 95%CI showing the effect of **a)** species, **b)** season.

Saliva samples

Saliva samples taken from positive greys were all negative for squirrelpox virus DNA (Table 4). The positive red from Tollymore Forest had a high level of pox virus infection.

Table 4. Viral load in saliva samples obtained from squirrelpox positive grey squirrels.

Species	Squirrel ID	qPCR result	Viral Load (v/s)
Grey	Ballykelly 3	Negative	0
	Ballykelly 4	Negative	0
	Crawfordsburn 3	Negative	0
	Drumbanagher 3	Negative	0
	Drumbanagher 9	Negative	0
	Drumbanagher 14	Negative	0
	Derrynoyd 7	Negative	0
	Killynether 3	Negative	0
	Killynether 5	Negative	0
	Killynether 6	Negative	0
	Loughgall 10	Negative	0
	Tollymore 6	Negative	0
Red	Tollymore Red 5	Positive	29,549,584

Urine samples

The lip and blood of 38 squirrels from which urine was successfully extracted were all negative for squirrelpox virus (Table 5). However the urine samples from 2 squirrels, both from Lissan, tested positive for squirrelpox virus DNA with a virus load of 1040 v/ml and 880 v/ml.

Table 5. Summary of ELISA and qPCR results. Values represent the numbers of squirrels (of both species combined).

	qPCR		
	-ve	+ve	Total
Squirrel Tissue	38	0	38
Urine	36	2	38

Faecal samples

All faeces samples from grey squirrels appeared normal and were negative for squirrelpox virus when analysed by qPCR. The faecal material from the infected red was black in colour (which may indicate the presence of blood) and liquid in appearance suggesting diarrhoea. The faeces from the infected red squirrel had a moderate level of squirrelpox virus (Table 6).

Table 6. Viral load in faeces samples obtained from squirrelpox positive grey and red squirrels.

Species	Squirrel ID	qPCR result	Viral Load (v/mg)
Grey	Ballykelly 4	Negative	0
	Ballykelly 7	Negative	0
	Crawfordsburn 3	Negative	0
	Drumbanagher 3	Negative	0
	Drumbanagher 9	Negative	0
	Drumbanagher 14	Negative	0
	Derrynoyd 7	Negative	0
	Killynether 3	Negative	0
	Killynether 5	Negative	0
	Killynether 6	Negative	0
	Loughgall 10	Negative	0
	Tollymore 6	Negative	0
Red	Tollymore Red 5	Positive	18,434

Identification of potential vectors

Ectoparasites occurred on 144 (70%) of 208 grey squirrels and 9 (23%) of 40 red squirrels. Fleas were the most abundant parasite, found on 69% of grey squirrels and 18% of reds squirrels. Ticks were present on 2% of grey squirrels and 13% of red squirrels, whilst mites occurred on 3% of grey squirrels but on no red squirrels. All parasites collected from the infected red tested positive using qPCR for virus DNA, while 27% of all fleas tested from infected greys had virus present (Table 7). Viral loads were relatively low for ectoparasites on grey squirrels [7 – 41 virus particles per ectoparasite v/e] in comparison to ectoparasites on the one infected red squirrel from Tollymore Forest Park (210 - 70,535 v/e). Ectoparasites were negative for the viral DNA if the squirrel host was also negative.

Table 7. Viral load in ectoparasites obtained from squirrelpox positive grey and red squirrels.

Species	Parasite type	Squirrel ID	Sample No.	qPCR result	Viral Load (v/e)
Grey	Flea	Ballykelly 4	1	Negative	0
		Ballykelly 4	2	Negative	0
		Crawfordsburn 3	1	Negative	0
		Crawfordsburn 3	2	Negative	0
		Drumbanager 3	1	Negative	0
		Killynether 5	1	Negative	0
		Killynether 5	2	Negative	0
		Loughgall 10	1	Negative	0
		Loughgall 10	2	Positive	7*
		Loughgall 10	3	Positive	41*
		Derrynoyd 7	1	Positive	26*
				Sub-mean (± 1SD)	**7 ± 14**
Red	Flea	Tollymore Red 5	1	Positive	210
		Tollymore Red 5	2	Positive	2,874
		Tollymore Red 5	3	Positive	9,480
				Sub-mean (± 1SD)	**4,188 ± 4,773**
	Tick	Tollymore Red 5	1	Positive	939
		Tollymore Red 5	2	Positive	2,162
		Tollymore Red 5	3	Positive	58,338
				Sub-mean (± 1SD)	**20,480 ± 32,792**
	Unknown	Tollymore Red 5	1	Positive	70,535
		Tollymore Red 5	2	Positive	25,683
		Tollymore Red 5	3	Positive	2,614
				Sub-mean (± 1SD)	**32,944 ± 34,538**
				Grand mean (± 1SD)	**9,909 ± 20,977**

* positive signal was detected however viral load was very low and falls outside of our ability to accurately calculate numbers.

Virus degradation rates

After one month of exposure to differing conditions in the laboratory, the number of degraded virus particles differed significantly between temperatures under both wet and dry atmospheres (Table 8a). More intact virus particles were observed in scabs kept in warm dry conditions than either cool or wet conditions (Fig. 8a). Similarly, the number of intact virus particles varied significantly between temperatures and conditions with the greatest number of intact particles surviving under warm dry conditions.

Table 8. Generalized Linear Models (GLMs) of the number of **a)** degraded and **b)** intact virus particles (virons) that remained after one month in wet and dry conditions at three temperatures.

Model / parameters	χ^2	df	*p*
a) No. of degraded particles			
Temperature	**33.658**	**2**	**<0.0001*** **
Wet/Dry	3.339	1	0.0680
Temperature*Wet/Dry	**21.540**	**1**	**<0.0001*** **
b) No. of intact particles			
Temperature	**17.924**	**1**	**<0.0001*** **
Wet/Dry	**15.931**	**1**	**<0.0001*** **
~~Temperature*Wet/Dry*~~	-	-	-

* Interaction could not be fitted as there were no particles under either wet or dry conditions at 15oC.

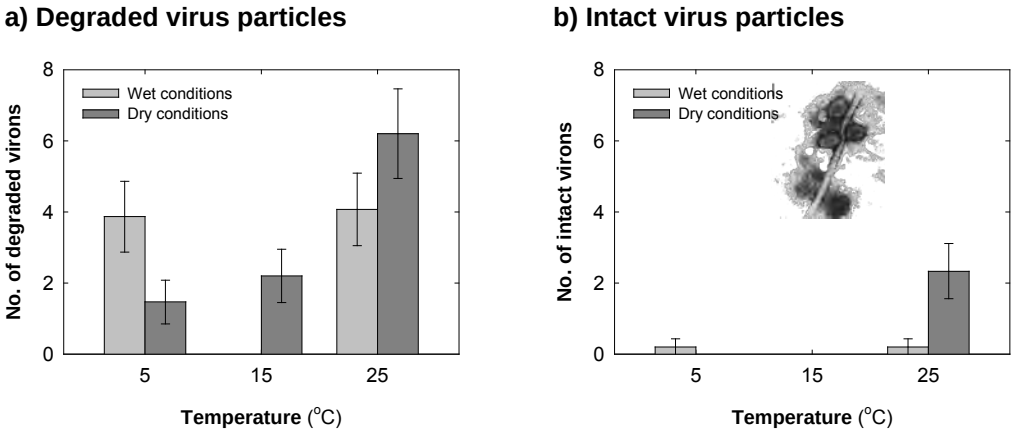

Figure 5. Degradation of the virus in wet and dry conditions at three temperatures after one month. Intact virus particles remained only in dry conditions at the highest temperature (25°C).

Power Analysis

A total sample of approximately *n*=800 would be required to detect a small (10%) change in the prevalence of SQPV throughout Northern Ireland. A moderate change (30%) could be detected with *n*=90 whilst a large change (50%) could be detected with as little as *n*=30 (Fig. 6). With the sample sizes at each locality in the current study (*n*= 4-32) only a large change in prevalence could be detected *within* sites during any future survey. However, if the current survey was repeated (i.e. with a similar *total* sample size of *n*=248) we might expect to be able to detect a 12.5% change in the prevalence of the disease throughout Northern Ireland as a *whole*.

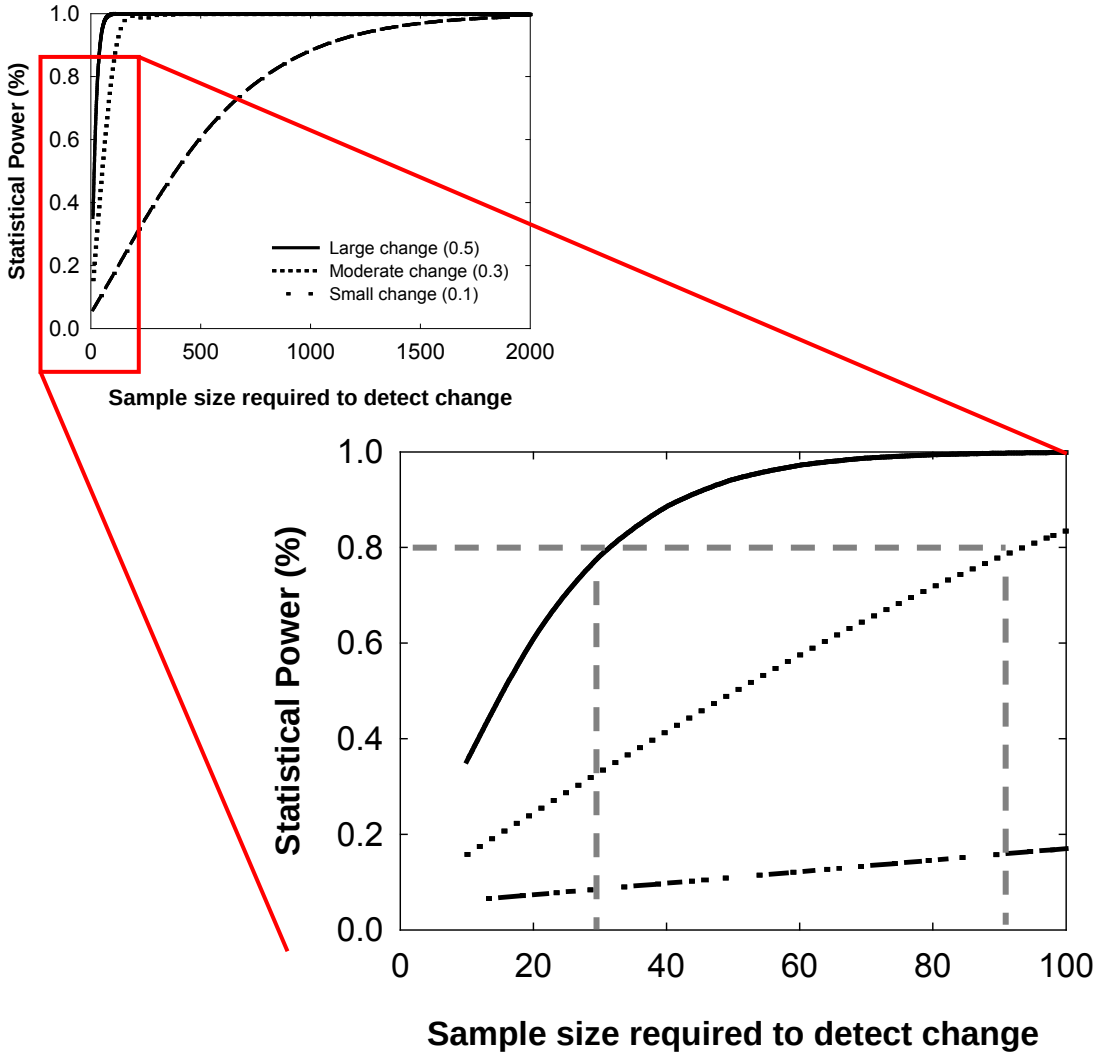

Figure 6. Power Analysis for a 2x2 χ^2 contingency table to test the change in prevalence of SQPV (number of positives verse number of negatives) between two survey periods (i.e. the current study and any future study). **a)** Change in statistical power with varying sample sizes for detecting a small, moderate and large change in prevalence (10%, 30% and 50% respectively). **b)** Insert of the same graph demonstrating that a sample size of *n*=30 squirrels would be required to detect a large (50%) change in prevalence whilst *n*=90 would be required to detect a moderate (30%) change.

DISCUSSION

The distribution of red and grey squirrels as described in the current study was similar to a recent All-Ireland squirrel survey published in 2007 (Carey *et al.* 2007), however, grey numbers have increased significantly during that time, particularly in mixed areas. Grey squirrels were widespread throughout Northern Ireland, including the Glens of Antrim which was only recently invaded (est. 2005). Red squirrels, whilst widespread, occur locally with 10km squares within which red squirrels occur in allopatry are typically isolated or surrounded by mixed populations (Fig. 1). It is likely that the distribution of the grey squirrel has been underestimated, as common species are typically reported less often than notably rare species.

Notwithstanding variation in the prevalence of the virus between sampled tissues, the number of grey squirrels infected with pox was relatively low at 8% at the time of sampling. The majority of grey squirrels infected were found in those forests designated as mixed red/grey populations (13 of 18 virus positive greys), including Killynether Wood, Co Down and Baronscourt Estate, Co. Tyrone, which had infection rates >30%. A much larger proportion of squirrels (22%) tested positive for squirrelpox antibodies, indicating past infection. Most of these came from areas of grey only populations (34 of 54 antibody positive greys). The distribution of seropositve squirrels was widespread; most forests with greys were positive for pox antibodies. However, several including Belvoir Forest Park, Redburn, Crawfordsburn, Killynether Wood and Castlearchdale had no past history of pox infection. The absence of antibodies but the presence of virus in Killynether Wood, Crawfordsburn and Castlearchdale suggests the disease has recently spread to these areas.

The disparity between the relative amount and location of the pox virus may be due to the immunity of grey populations; greys from areas with a high level of seropositivity had a lower level of squirrelpox virus, perhaps due to some kind of “herd immunity” effect (John & Samuel, 2000). In contrast, squirrels from populations with a low number of seropositive individuals had some of the highest levels of pox virus. Several of these forests occur at red/grey interfaces, most notably Killynether Wood in the Newtownards area which was just 8km north-west of Mount Stewart,

which hosts one of the few remaining red squirrel populations effectively isolated on the Ards Penninsula. The presence of greys with a high prevalence of squirrelpox virus poised to invade the Peninsula poses a substantial risk to the red squirrels at Mount Stewart. In addition, the Ards peninsula hosts red squirrel populations in Greyabbey, Kircubbin and Portraferry, all of which are threatened should greys and SQPV move south, as already appears to be happening (Jaimie Dick *pers. obs.*).

Most squirrels with pox virus DNA were negative for squirrel pox antibodies suggesting that at the time of testing they were in the earlier stages of infection before any host antibody immune response could develop. Those few squirrels that had both pox antibodies and pox virus DNA may currently be in the process of virus clearance. Only one red squirrel examined was positive for pox virus or antibodies. This individual was collected at Tollymore Forest park, Co. Down and presented a very high level of active infection with thick scabs on the lower lip and severe dermatitis. No other reds tested positive for the disease supporting the notion that most reds which contract SQPV die from the infection (Sainsbury *et al*. 2000).

Bruemmer *et al.* (2010) found that the presence of squirrelpox antibodies differed between the sexes and was positively correlated with Autumn/Winter. Males typically have larger home ranges than females and move further distances, increasing the likelihood of encountering the disease, whilst testosterone may lower their immune response making them more vulnerable to infection (Klein, 2000; Moore and Wilson, 2002). Our results conflict with those of Bruemmer *et al.* (2010) as field observations suggested that the prevalence of the virus was greatest in spring and summer whilst lowest in winter. Indeed, electron microscopy of pox virus particle degradation, suggested that the virus survived longer in warm dry conditions than in cool wet conditions. Spring and summer are also likely to favour vector-borne (ectoparasite) transmission. Indeed, several ectoparasite species examined, including fleas and ticks that occurred on infected squirrels, hosted the virus supporting previous suppositions that they may act as vectors (LaRose *et al.,* 2010; Atkin *et al.,* 2010; McInnes et al., 2012). The presence of virus DNA in the faeces and saliva from the positive red squirrel from Tollymore Forest Park, Co. Down and its absence from the faeces and saliva of positive grey squirrels suggests that environmental spread of the virus may be a viable route of transmission in red squirrels (due to their high viral

load and expression of diarrhoea-like symptoms as observed in the infected red squirrel from Tollymore), but less likely in grey squirrels. The detection of virus DNA in the urine of two grey squirrels yet the failure to detect the virus DNA in either the blood or lip is surprising, however, rodents are known to pass several viruses in their urine (Bharti *et al*. 2003; Kallio *et al*. 2006) and squirrelpox may be passed between greys in a similar manner. Thus, whilst the most likely vector for the disease appears to be ectoparasites we cannot rule out a faecal route in red squirrels or urine in grey squirrels.

Squirrelpox virus is indigenous to grey squirrels and is present in most populations throughout Northern Ireland. However, grey squirrels typically have low viral loads and recover from the disease. Forests with no history of squirrelpox infection (i.e. squirrels lacking antibodies) but where virus has been detected, appear to occur at interface areas between grey and red squirrel populations and pose the greatest epidemiological risk to nearby red populations. Killynether Wood, with its close proximity to the red population at Mount Stewart and the broader Ards peninsula, is particularly notable in this respect. Limiting the contact between the two species in this area, and others such as Ballycastle, Baronscourt and Castlearchdale, may provide one means by which to slow or prevent the spread of the disease to remaining red squirrels. In areas where the range of the two species overlaps, measures to reduce encounter rates are considered essential (McInnes *et al*. 2012).

RECOMMENDATIONS

We make 5 recommendations:

Conservation action

1. Surveillance of squirrelpox within red and grey squirrels in areas where the range of the two species overlap.

2. Establishment of buffer zones around remaining red squirrels perceived as vulnerable to infection (for example, Mount Stewart, Ards Peninsula, Ballycastle and East Fermanagh). Grey squirrels should be trapped or shot to limit contact with nearby red squirrels.

Further research

3. A focal study of a number of mixed populations (for example, Greater Belfast, Ballycastle or Newtownards) and high prevalence grey only populations (for example, Derrynoyd or Portglenone) may yield greater immunological insights provided higher intensity sampling can be maintained at study sites (as determined by Power Analysis).

4. A focal study in a seronegative population(s) which appear to harbour squirrel pox to investigate infectious disease dynamics.

5. A focal study of vulnerable red squirrel populations (for example, Mount Stewart, Tollymore, Baronscourt or East Fermanagh) using capture-recapture to repeatedly sample individuals for virus DNA or antibodies to provide early detection of infection and allow greater immunological study of the disease in red squirrels.

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REFERENCES

Atkin, J.W., Radford, A.D., Coyne, K.P., Stavisky, J., Chantrey, J., 2010. Detection of squirrelpoxvirus by nested and real-time PCR from red (*Sciurus vulgaris*) and grey (*Sciurus carolinensis*) squirrels. *BMC Veterinary Research*, **6**:33.

Bharti, A,R., Nally, J,E., Ricaldi, J,N., Matthias, M,A., Diaz, M,M., Lovett, M,A., Levett, P,N., Gilman, R,H., Willig, M,R., Gotuzzo, E., Vinetz, J,M., 2003. *Leptospirosis*: a zoonotic disease of global importance, *The Lancet Infectious Diseases*, Volume 3, Issue 12, , Pages 757-771.

Bruemmer, C. M., Rushton, S. P., Gurnell, J., Lurz, P. 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. Epidemiology and Infection, 138, 941-950.

Carey, M., Hamilton, G., Poole, A. and Lawton, C. 2007. The Irish squirrel survey. COFORD, Dublin.

Duff, J.P., Scott, A., Keymer, I.F., 1996. Parapox virus infection of the grey squirrel**.** *Veterinary Record* 138(21):527.

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

Gurnell, J., 1996. The grey squirrel in Britain: problems for management and lessons for Europe. *European Mammals* (eds M.L.Mathias,M.Santos-Reis, G.Amori, R.Libois, A.Mitchell-Jones & M.C.Saint-Girons), pp. 67 – 81.

Gurnell, J., Wauters, L. A., Lurz, P. W. W. and Tosi, G., 2004. Alien species and interspecific competition: effects of introduced eastern grey squirrels on red squirrel population dynamics. *Journal of Animal Ecology* 73; 26–35.

Kallio, E. R., Klingstrom, J., Gustafsson, E., Manni, T., Vaheri, A., Henttonen, H., Vapalahti, O., and Lundkvist, A., 2006. Prolonged survival of *Puumala hantavirus* outside the host: evidence for indirect transmission via the environment. *Journal of General Virology*, 87:2127-2134.

Klein, S.L., 2000. The effects of hormones on sex differences in infection: from genes to behavior. *Neuroscience & Biobehavioral Reviews.* 24,627–638.

LaRose, J.P., Meredith, A.L., Everest, D.J., Fiegna, C., McInnes, C.J., Shaw, D.J., Milne, E.M., 2010. Epidemiological and postmortem findings in 262 red squirrels (*Sciurus vulgaris*) in Scotland, 2005 to 2009. *Veterinary Record* 2010;167:297-3

Livingston, C.W., Hardy, W.T., 1960. Longevity of contagious ecthyma virus. *Journal of American Veterinary Medical Association* 137:651.

John, T.J., Samuel, R., 2000. Herd immunity and herd effect: new insights and definitions. *European duffJournal of Epidemiology* 16*:* 601–606.

McInnes, C. J., Wood A. R., Thomas K., Sainsbury A. W., Gurnell J., Dein F. J., Nettleton P.F., 2006. Genomic characterization of a novel poxvirus contributing to the decline of the red squirrel (*Sciurus vulgaris*) in the UK. *Journal of General Virology* 87(8):2115 - 2125.

McInnes, C. J., Coulter, L., Dagleish, M. P., Deane, D., Gilray, J., Percival, A., Willoughby, K., Scantlebury, M., Marks, N., Graham, D., Everest, D. J., McGoldrick, M., Rochford, J., McKay, F., Sainsbury, A. W., 2012. The emergence of squirrelpox in Ireland. *Animal Conservation*. doi: 10.1111/j.1469-1795.2012.00570.x

Middleton, A.D., 1930. The ecology of the American grey squirrel (*Sciurus carolinensis*) in the British Isles**.** *Proceedings of the Zoological Society of* London 2:809-843.

Moore, S.L. & Wilson, K., 2002. Parasites as a viability cost of sexual selection in natural populations of mammals. *Science* 297,2015–2017.

Rushton, S.P., Lurz, P.W., Gurnell, J., Nettleton, P., Bruemmer, C., Shirley, M.D., 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**.** *Epidemiology and Infection*, 134(3):521-533.

Sainsbury, A. W., Nettleton, P., Gilray, J. and Gurnell, J., 2000. Grey squirrels have high seroprevalence to a parapoxvirus associated with deaths in red squirrels. *Animal Conservation*, 3: 229–233.

Sainsbury, A.W., Deaville, R., Lawson, B., Cooley, W.A, Farrelly, S.S.J., Stack, M.J., Duff, P., McInnes, C.J., Gurnell, J. and Russell, P.H., 2008. Poxviral Disease in Red Squirrels Sciurus vulgaris in the UK: Spatial and Temporal Trends of an Emerging Threat. *Ecohealth* Volume 5, Number 3, 305-316.

Scantlebury, M., Maher McWilliams, M., Marks, N. J., Dick, J. T. A., Edgar, H. and Lutermann, H., 2010. Effects of life-history traits on parasite load in grey squirrels. *Journal of Zoology*, 282: 246–255.

Scott, A.C., Keymer, I.F. & Labram, J., 1981. Parapoxvirus infection of the red squirrel (*Sciurus vulgaris*). *Veterinary Record*, 109, 202.

Sweet, D., Lorente,M., Lorente, J.A., Valenzuela, A., and Villanueva, E.,1997. An Improved Method to Recover Saliva from Human Skin: The Double Swab Technique. *Journal of Forensic Science*, 42(2):320-2.

Tompkins, D.M., Sainsbury A.W., Nettleton P., Buxton D., and Gurnell J., 2002. Parapoxvirus causes a deleterious disease in red squirrels associated with UK population declines. *Proceedings of the Royal Society of London, Series B*; 269: 529–533.

Wauters, L. A. and Gurnell, J., 1999. The Mechanism of Replacement of Red Squirrels by Grey Squirrels: A Test of the Interference Competition Hypothesis. *Ethology*, 105: 1053–1071.

APPENDIX 1 – Questionnaire distributed to conservationists, local rangers and park official to assess squirrel distribution

1. Name .....

2. Area/region in which you work (eg county, name of forest(s))....

3. How often do you visit the area .....

4. What is your role
(Squirrel group, ranger etc) .....

5. Have you seen any RED squirrels in this area? Yes No

6. Have you seen any GREY squirrels in this area? Yes No

7. During the last year, what has been the maximum number of squirrels you have seen in one day?

Red_____ Grey_____

8. How do the number of squirrels observed this year compare with previous years:

	Red			Grey		
	Lower	Similar	More	Lower	Similar	More
2011						
5 years ago						

9. How regularly do you see squirrels? Red Every Day Once a Week Once a Month

Grey Every Day Once a Week Once a Month

10. Do you generally see squirrels in the same general area? Red Yes No

Grey Yes No

If yes:

a) Grid Reference: _____ (map)

b) Post Code: _____ (internet)

11. Do the managers of the locations where you see squirrels provide food for them?

Yes No

12. Do you suspect that any squirrels you observe may have disease?

Yes No

If yes, what symptoms did you observe?_____

13. Specifically, have you ever seen any squirrels with lesions (open abscesses, discolouration of fur)?

Yes No

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