The drivers of squirrelpox virus dynamics in its grey squirrel reservoir host

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ABSTRACT

Many pathogens of conservation concern circulate endemically within natural wildlife reservoir hosts and it is imperative to understand the individual and ecological drivers of natural transmission dynamics, if any threat to a related endangered species is to be assessed. Our study highlights the key drivers of infection and shedding dynamics of squirrelpox virus (SQPV) in its reservoir grey squirrel (*Sciurus carolinensis*) population. To clarify SQPV dynamics in this population, longitudinal data from a 16-month mark-recapture study were analysed, combining serology with real-time quantitative PCR to identify periods of acute viraemia and chronic viral shedding. At the population level, we found SQPV infection prevalence, viral load and shedding varied seasonally, peaking in autumn and early spring. Individually, SQPV was shown to be a chronic infection in > 80% of grey squirrels, with viral loads persisting over time and bouts of potential recrudescence or reinfection occurring. A key recurring factor significantly associated with SQPV infection risk was the presence of co-infecting squirrel adenovirus (ADV). In dual infected squirrels, longitudinal analysis showed that prior ADV viraemia increased the subsequent SQPV load in the blood. However, there was a strong, negative association between prior ADV viraemia and subsequent SQPV shedding from the forearm, probably caused by ADV prolonging the SQPV acute viraemic phase, so delaying onset of the chronic shedding phase, and thereby altering viral shedding patterns over the time scales examined here. Hence, co-circulating ADV infection may be involved in mediating both the quantitative levels of SQPV infection and the timing and degree of subsequent infectiousness of grey squirrels.

1. Introduction

Many pathogens of conservation concern circulate endemically within reservoir host populations, before spilling over with potentially devastating consequences to a target host species of conservation or human interest (Haydon et al., 2002; Fenton and Pedersen, 2005; Lloyd-Smith et al., 2009). Classic examples include Ebola emerging from bats, chytridiomycosis circulating within many amphibian communities globally, rabies in a variety of mammalian species, and canine distemper virus in large carnivores in the Serengeti (Fisher and Garner, 2007; Viana et al., 2015; Goldstein et al., 2018). In all these cases, the pathogen circulates within the reservoir host population, often with little obvious effect (Hazel et al., 2000; Knowles et al., 2010), but upon exposure to a naïve host population it can cause marked disease mortality, so much so that the new host may be unable to maintain the infection without its natural reservoir (Fenner, 1953; Prager et al., 2012; Fenton et al., 2015). In many cases, the timing and/or location of the spillover event can be highly unpredictable, making robust

preventative management particularly challenging. To facilitate these predictions an understanding of the dynamics of the pathogen within its reservoir population, and identification of key drivers of pathogen transmission at the individual, population and environmental levels are required (Lloyd-Smith et al., 2009; Viana et al., 2014). Identifying these factors accurately requires longitudinal studies whereby individuals are tracked throughout their lives, and changes in infection status can be quantified and related to those individual, population and environmental factors. However, for most microparasitic pathogens (e.g., viruses, bacteria) infection status is typically recorded as presence or absence of detectable infection, whereas it is increasingly recognised that changes in pathogen load (e.g., the quantitative levels of viraemia or bacteriaemia) are key determinants of the rate of pathogen transmission within the reservoir population (McCallum et al., 2017), and hence risk of spilling over to the target species. Clearly however, gathering such high-resolution data for many wildlife reservoir species is logistically challenging, and hence very rare. Here we present a longitudinal study into the drivers underlying infection risk and

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infectiousness for a pathogen of major conservation concern within its natural wildlife reservoir.

The introduced grey squirrel (*Sciuris carolinensis*) in the UK is considered to be the reservoir host for squirrelpox virus (SQPV), a virus pathogenic to the native red squirrel (*Sciuris vulgaris*) that has been implicated in dramatic population declines nationally (Sainsbury et al., 2000; Chantrey et al., 2014). Both field (Chantrey et al., 2014) and modelling studies (Tompkins et al., 2003; Rushton et al., 2006) have identified squirrelpox virus as a major threat to the remaining red squirrel populations in the UK. Squirrelpox (SQPx), the clinical disease in red squirrels, has been linked to infection in grey squirrels (Chantrey et al., 2014) and, given the devastating impact SQPx has in red squirrel populations, which offers limited opportunities for sustained transmission among red squirrel populations alone, it is highly likely that grey squirrels are the maintenance reservoir for infection. As such, it is vital to understand the infection dynamics of SQPV in this reservoir population to predict future disease outbreaks in the red squirrel population and so develop preventative measures that limit the impact on endangered UK native squirrels.

Currently, little is known about SQPV pathogenesis or epidemiology, especially within the reservoir grey squirrel population. The virus is proposed to be restricted to cutaneous tissue in the grey squirrel after transient periods of viraemia. Only rare individuals show any discernible skin lesions (Atkin et al., 2010; Bruemmer et al., 2010). Viral DNA has been identified from various organs and excreta but the highest viral loads are found in the skin, especially the arm vibrissal nodule (Dale et al., 2016). Environmental contamination is still proposed to be the most important route of transmission (Sainsbury et al., 2008; Bruemmer et al., 2010), although both empirical (Atkin et al., 2010; LaRose et al., 2010) and modelling studies have suggested a role for fleas in the transmission of SQPV (Cowan et al., 2016). Co-infection with other microparasites could also play a role in infection dynamics, as has been shown to be important in other rodent species (Telfer et al., 2010; Knowles et al., 2013). In particular, squirrel adenovirus (ADV) has been suggested to be of potential significance to UK squirrel populations. Although not directly implicated in population declines, ADV infections have been reported in red squirrels with increasing frequency, all identifying enteritis as the primary lesion (Duff et al., 2007; Everest et al., 2008, 2010a; Martinez-Jimenez et al., 2011; Everest et al., 2012), and have been shown to cause mortality in individual red squirrels (Everest et al., 2010b). Evidence for adenoviral infection in grey squirrels is sporadic; it has been identified in healthy grey squirrels (Everest et al., 2009), but the majority of cases are associated with red squirrel re-introduction programs (Martinez-Jimenez et al., 2011; Everest et al., 2012). Antibodies to ADV were first detected in grey squirrel blood (Greenwood and Sanchez, 2002) but an investigation of a cluster of ADV-related red squirrel deaths, during a translocation program, detected no ADV infection in the surrounding grey squirrel population (Martinez-Jimenez et al., 2011). Furthermore, infections are seen in red squirrels in areas, such as off-shore islands, entirely lacking grey squirrels (Everest et al., 2013). However, there is growing evidence to suggest a large proportion of grey squirrels may have subclinical infections (Everest et al., 2012), although much of our ADV knowledge to date is based on host mortality data, and little is known about its epidemiology in free-living squirrel populations, or possible associations with SQPV infections.

Clearly, there is a need to disentangle the intrinsic and extrinsic drivers of SQPV dynamics in natural grey squirrel populations, including the role of possible interactions with coinfecting ADV. To date, however, our understanding of SQPV transmission and epidemiology in grey squirrels has been hampered by the type and resolution of data available from wild populations. Previous epidemiological studies of SQPV infection in grey squirrels have either used squirrels culled in control programs, and/or SQPV infection status has been quantified based on serology (Sainsbury et al., 2000; Bruemmer et al., 2010). Culled grey squirrels are a useful source of information, but they only

give a single point prevalence at the time of the cull, with no information about the longitudinal disease dynamics in individuals. In addition, serology gives a history of an animal's previous viral exposure only, rather than ongoing infection, so providing little information about when viral exposure occurred or whether the infection is still active with the potential to transmit onwards from that individual. To clarify the drivers of infection and transmission dynamics typically requires fine resolution, longitudinal data based on repeat captures of multiple individuals in the population (Fenton et al., 2014). Fortunately, recently developed molecular techniques now facilitate the detection and quantification of both SQPV and ADV viruses in live individuals (Dale et al. 2016), enabling unprecedented insight into the quantitative associations and interactions between them. Here we apply these techniques to samples from a longitudinal mark-recapture study of a wild grey squirrel population to quantify the dynamics of SQPV infection in its natural reservoir population, and identify the drivers, including ADV co-infection, associated with infection risk and load of SQPV infection.

2. Methods

2.1. Fieldwork

Live squirrel trapping was conducted for 3 consecutive days, at least once per month from September 2009 until December 2011 at Ness Botanical Gardens (Ness, Cheshire, UK) (OS ref: 117:SJ305755). The study site comprised a grid of 200 m square quadrants with two trap pairs randomly sited in each quadrant, giving an overall trap density of 1 trap per hectare (40 traps in total). Traps were baited, set around sunrise and were sequentially checked 3–4 hours later. No traps were left set overnight.

Morphometric data were recorded from each trapped squirrel, which included weight, body condition, age, sex and tibial length. The tibial measurement was included as a secondary parameter, in addition to external reproductive appearance (Appendix S1), for age (Morris, 1972), which can be an imprecise measurement. The degree of any ectoparasite infestation (for further detail see supplemental material) and any skin abnormality (including any ulceration, scabbing or inflammation to the integument) were also recorded. Individual squirrels were identified using PIT tags (AVID Plc., Surrey, UK) placed subcutaneously when first captured. A 1 ml blood sample was taken from the greater saphenous vein, from which the serum and cell pellet were separated within twelve hours of collection and stored at −20 °C. A Dacron swab was taken from each of the following 4 sites: the oral cavity, periocular, forearm and rectum. Skin was examined for the presence of ectoparasites, which were graded (as per supplemental material). All grey squirrels were released after sampling, permitted under a Natural England licence. All traps in which a squirrel was caught were cleaned of organic debris and disinfected by submersion in 2% Trigene® (Medichem International Ltd., Kent, UK) prepared using the manufacturer's recommendations, for a minimum of 30 min. After submersion, traps were rinsed with fresh water and returned to their original position. During non-trapping weeks, traps remained in their field locations with doors locked open and were baited approximately twice weekly.

2.2. SQPV serology and multiplex SQPV and ADV qPCR

An ELISA was used to detect SQPV antibodies in blood sera (Sainsbury et al., 2000). As SQPV behaves similarly to other pox viruses, being cell-associated when present in the blood (Bennett et al., 1989; Nitsche et al., 2007), the cell pellet, present after whole blood centrifugation and serum separation, was used to determine the viraemic status of both viruses. Cutaneous/mucocutaneous swabs (vibrissal arm nodule, eyelid and oral cavity) were used to determine the infection/shedding status of SQPV and also rectal swabs for SQPV and

ADV. All the samples were analysed for viral DNA as per Dale et al. (2016).

2.3. Statistical analyses

We conducted analyses on two sources of SQPV data, one based on presence of SQPV viraemia by PCR from blood samples (n = 485 samples from 106 individuals), and the other on SQPV DNA from the arm swab samples (n = 248 samples from 29 individuals). These analyses were chosen as the former is a measure of an individual's acute infection status, where the squirrel is being systemically challenged by SQPV replication and dissemination. The latter is indicative of localised chronic infection and potentially acts as a major source of chronic viral shedding and hence the potential for onward transmission from that individual. For each source of data (blood or arm swabs), we conducted two forms of analysis: one examining the factors (see below) associating with an animal showing positive SQPV infection (i.e., analysis of infection risk), and the other examining the factors associated with the quantitative infection load (by qPCR) among SQPV-positive animals (i.e., analysis of infection intensity).

For all analyses, factors involved in acute or chronic infection with SQPV (presence of viraemia in blood samples or virus in arm swabs) were investigated through mixed model regression analysis, either with a binomial error structure (with a log link function) for analyses of infection risk, or a Gaussian error structure on logged data for analyses of infection intensity. All GLMM analyses were performed using either the R package AD Model Builder (*glmmADMB*) (Bolker et al., 2009; Fournier et al., 2011) or the package *lmer* if *glmmADMB* failed to converge. Model selection was based on the Akaike information criterion (AIC). For an effect to remain in a model it was required to cause a reduction in AIC (ΔAIC > 2); otherwise the model with fewer parameters was selected (Sakamoto et al., 1986). Individual squirrel identity was included as a random effect to control for pseudoreplication arising from repeat captures of the same individual.

We included similar sets of factors in all analyses, and in each case model development was carried out in a stepwise manner, in which an overall model was built through a series of three phases, to test specific hypotheses of fixed effects and selected interactions; the minimal model at the end of each phase was used as the starting point for the subsequent phase. The first phase only considered contemporary biometric measurements as fixed effects: tibia length, weight, age, sex, reproductive status, body condition score (BCS; details in supplemental material) and the presence or absence of wounds/skin lesions, plus the individual trap in which the squirrel was caught and whether it had been captured before, to check for any evidence that the trapping activity may have facilitated the transmission of SQPV. We also included a temporal component to account for seasonal variation in infection risk; for the analysis of blood PCR data this was a monthly variable as a factor (11 levels: March was excluded as no infections were observed in this month, which made the model unstable), whereas for the arm swab data, due to sample size limitations, we used a 'season' variable (4 levels: winter [December – February inclusive], spring [March – May], summer [June – August], autumn [September – November]). We also explored pairwise interactions among certain variables (weight, tibia length, age, sex, reproductive status) to test hypotheses relating to SQPV infection risk (e.g., whether reproductively active, or larger, males were at high risk of infection (Sainsbury et al., 2000; Bruemmer et al., 2010)).

After model simplification at this first phase (retaining all factors and/or interactions with ΔAIC > 2), we then used the resulting minimal model as the baseline to test for associations between serological status and viraemia (both as seropositivity and Optical Density [OD] values) with both contemporary measures, and previous and subsequent change in OD measurements between successive captures. We also explored the effects of previous and subsequent changes in individual BCS score and weight to investigate the hypotheses that previous

physiological stress may lead to increased SQPV infection rates, while SQPV infection may be associated with subsequent weight loss or a reduction in body condition. In addition, associations with the presence or absence of skin abnormalities were also investigated, hypothesising that mild skin lesions may be associated with SQPV infection, given that severe lesions are found in infected red squirrels. To assess whether the arm swab data were an accurate representative of other swab data, we also sought associations between SQPV detected in arm swab data and virus detected from oral, rectal and periocular swabs.

Finally, we built on the minimal model from the previous two phases as a starting point to assess associations between SQPV infection status and infection with ectoparasites (fleas and lice; scored either as presence/absence, absolute count, or a grade dependent on the count) and ADV (presence/absence). It was hypothesised that if ectoparasites were vectors of SQPV, then previous presence and/or high burdens of ectoparasites would be associated with subsequent SQPV infection. Hence, contemporary, previous and subsequent associations of ectoparasites with SQPV infection status were explored. For ADV, we hypothesised that the immunosuppressive effect of SQPV may potentially lead to an increase in infection rates or viral load of the other, so we included past, contemporary and future infection status with ADV in our models; incorporating such lagged effects has previously been shown to be a more reliable approach to identifying potential interactions between coinfecting parasites, than using purely contemporary (cross-sectional) analyses of parasite co-association, which are unable to disentangle true interaction from potential confounding factors (Fenton et al., 2014). At the end of this third phase of model development, all remaining terms were finally assessed for inclusion, based on whether their removal resulted in an increase of more than 2 AIC units. This resulted in the final models for each analysis presented below.

3. Results

3.1. Population- and individual-level infection dynamics

There were 720 captures leading to 511 samples (from squirrels caught for the first time each trapping session). Samples per squirrel ranged from 1 to 16 (mean = 4.44). At the population level, SQPV seroprevalence remained high (> 60%) throughout the sampling period, but showed successive dips in October/November each year (Fig. 1A, Fig S1). SQPV prevalence based on viraemia, however, tended to be much lower (0–60%; Fig S1), fluctuating considerably, but showing a gradual decrease throughout the sampling period (Fig. 1B). ADV viraemia showed broadly similar seasonal trends to SQPx viraemia, tending to be low in prevalence (0–40%; Fig S1), and again showing a gradual decline throughout the sampling period (Fig. 1C).

At the individual level, squirrels varied in their fluctuations of serological titre and pox viraemia across successive captures, with some just showing single peaks, and other showing repeated increases/decreases sometimes several months after their initial seroconversion (see Supplementary Material Appendix S2). While second viraemias were usually of lower magnitude when compared with the first, this was not always the case for excretion of viral DNA from cutaneous arm swabs, with a few seroconverted individuals showing comparable peaks in cutaneous shedding to those of the initial infection. SQPV DNA shedding occurred at all the body sites examined (arm, periocular, rectum, oral cavity) but the amount of virus recovered was most sustained and of greatest magnitude in the antebrachial (arm vibrissal nodule) swab throughout the time period studied. The duration of SQPV shedding from arm swabs was often chronic, with repeated peaks and troughs (see Supplementary Material Appendix S2).

3.2. Factors associated with SQPx viraemia (blood)

For analysis of the SQPV presence/absence from blood sample data, the final model included only a fixed effect of month and the
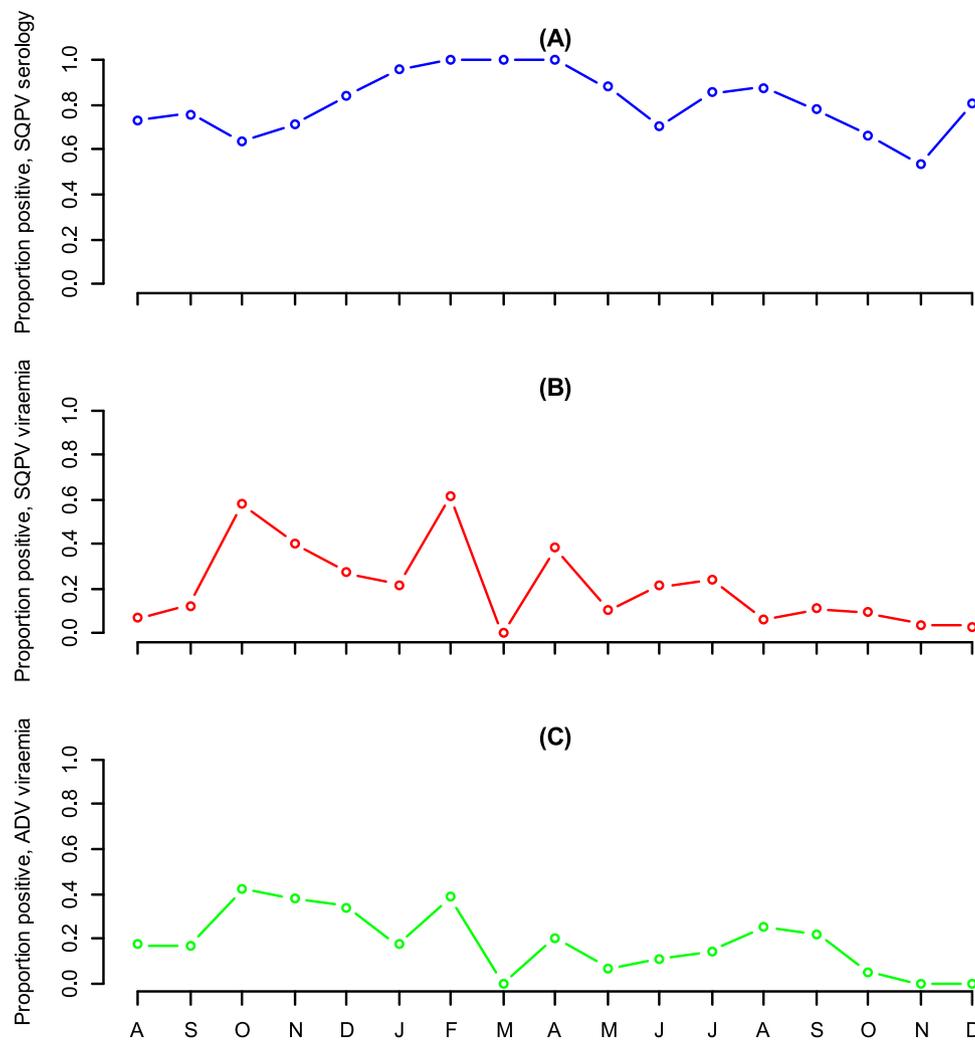
Fig. 1. SQPV prevalence over 16 months as determined by (A) serology and (B) QPCR analysis of blood samples. (C) ADV prevalence as determined by qPCR of blood samples.

contemporary (same capture session) presence of adenoviraemia (Table 1). SQPV prevalence was highest in February (coefficient relative to January (baseline) = 1.60) and was lowest in March (no infections recorded, and was excluded from the analysis due to model instability). Including contemporary ADV infection status improved the model considerably (ΔAIC = -45.6, coefficient 1.797, Odds Ratio = 6.03) showing hosts with adenoviraemia in their blood were six times more likely to also have detectable SQPV in their blood compared to hosts without ADV. However, no improvement was seen with the inclusion of adenoviraemia at the previous or subsequent capture.

ADV status associated positively with SQPx viraemic load among SQPV-positive animals, both at the current trapping session (contemporary effect; coefficient relative to ADV-negative animals = 0.86; Odds Ratio = 2.36; Table 2; Fig. 2) and from the previous trapping session (coefficient = 0.50; Odds Ratio = 1.64; Fig. 2). Hence, although previous ADV infection had no effect on the probability of an animal subsequently becoming SQPV infected (i.e., no effect on host susceptibility; see above), it did have a contributory effect on the intensity of SQPV infection in blood, among those animals that became infected. Notably, this lagged effect was not altered by including SQPV load at that previous trapping session in the model, suggesting this effect was not simply reflecting an on-going previous co-association of SQPV and ADV.

We also found relationships between SQPx viraemic load and both

past and future flea infestation (Table 2). SQPV-infected animals tended to have ~50% lower SQPV loads if they were found to have fleas at the previous capture, compared to animals without fleas at the previous capture (coefficient relative to flea-negative animals = -0.74; Odds Ratio = 0.48; Table 2). However, there was a positive association between SQPV load and the likelihood of having fleas at the subsequent capture (coefficient relative to flea-negative animals = 0.65; Odds Ratio = 1.91), suggesting high SQPx viral loads may in some way facilitate the probability of subsequently having fleas. Finally, there was a strong positive association between the presence of skin lesions and SQPx viraemic load (coefficient relative to animals with no visible lesions = 1.16; Odds Ratio = 3.20; Table 2), in addition to a strong seasonal pattern to SQPx viraemia among infected animals (being highest in October, and lowest in May; Table 2).

3.3. Factors associated with SQPV shedding (forelimb)

Analysis of the arm swab SQPV shedding data again revealed a strong seasonal association, with prevalence being highest in the winter (coefficient relative to autumn (baseline) = 1.95) and lowest in the summer (coefficient = -0.12; Odds Ratio = 0.89; Table 3). There was also a strong positive association between being SQPV-positive from arm swab samples and being SQPV-positive from oral swab samples (coefficient relative to SQPV-negative animals from oral samples = 1.251, Odds Ratio = 3.49; Table 3). In addition, animals that

Table 1
Mixed model analysis with presence or absence of SQPV viraemia from blood samples as the dependent variable. **(a)** Optimal model development - models that showed a reduction in AIC of over 2 units compared to the previous model. **(b)** Optimal model parameter estimates. The ΔAIC displayed is the change in AIC that occurred with the removal of the fixed effect from the optimal model. Abbreviations = SQPV = SQPV viraemia presence or absence from blood samples, SQ ID = individual squirrel identification number, ADVp = ADV infection status (positive/negative) at the same capture.

(a)

investigating	model; SQPV ˜ (1|SQ	n(obs)	n(t)	AIC	ΔAIC
factor	ID) +				
base model	month	485	106	513.90	–
ADV	month + ADVp	485	106	468.31	**−45.6**

(b)

SQPV ˜ (1|SQ ID) + month+ADVp

Factor	AIC	ΔAIC	Coefficient	SE	z-value
(intercept)	468.31		−1.709	0.543	−3.15
Month:	491.99	23.68			
February			1.599	0.691	2.32
April			0.853	0.642	1.33
May			−0.672	0.697	−0.96
June			0.145	0.722	0.20
July			0.208	0.763	0.27
August			−1.559	0.819	−1.90
September			−0.802	0.776	−1.03
October			0.847	0.604	1.40
November			0.181	0.608	0.30
December			−0.382	0.628	−0.61
ADV (positive)	513.90	45.60	1.797	0.265	6.77
SQ ID	466.31	−2			

had increased in weight since their previous capture were more likely to be SQPV shedding from arm swab samples (coefficient per unit increase in log body weight = 102.2). Finally, there was a weak positive association between the presence of SQPV from arm swabs and contemporary flea count (coefficient relative to flea-negative animals = 0.165; Odds Ratio = 1.18; Table 3), such that animals with more fleas were more likely to also be SQPV positive from arm swab samples.

There was a strong seasonal signal in SQPV infection load from arm swabs (being highest in winter and lowest in spring; Table 4), and there was a strong positive association between arm swab SQPV shedding levels and the increase in serology Optical Density (OD) from ELISA analysis of SQPV antibodies from the previous capture to the current one (coefficient per unit increase in OD = 19.76; Table 4; Fig S2), reflecting an increasing serological response to SQPV. Furthermore, SQPV shedding from arm swabs at one capture correlated with arm swab SQPV shedding at the previous capture (coefficient = 0.10; Table 3), implying sustained levels of shedding among infected animals. Interestingly, there was also a strong association with previous ADV infection status, and this effect was negative (coefficient = -079; Odds Ratio = 0.45; Table 4), implying that animals with previous ADV infection tended to have ˜50% lower SQPV shedding in their arm swab samples at the subsequent capture, compared to animals that did not have previous ADV infection (Fig. 3).

4. Discussion

This study is the first longitudinal epidemiological study of a wild grey squirrel population that has provided detailed information on the epidemiology of SQPV, and the factors leading to SQPV infection and shedding, in its endemic reservoir host. We found SQPV infection to occur at a high prevalence, typically causing chronic infection, with

repeating periods of recovery and recrudescence or reinfection despite a concurrent antibody response. Unsurprisingly, our analyses highlighted that there are multiple influences, including other microparasites that may have an effect on pathogen dynamics.

At the population level, we found SQPV seroprevalence was relatively high, at an overall figure of 80% but increasing to ˜100% in early spring. These findings support the theory that seroprevalence to poxviruses increases when naïve juvenile populations are at their peak resulting in outbreaks of infection (Chantrey et al., 1999). Previous studies have reported a seroprevalence of SQPV in grey squirrels of between 53 to 63% (Sainsbury et al., 2000; Bruemmer et al., 2010); although the latter study does note that prevalence tends to be higher in established grey populations with no culling. This is reinforced by data from Anglesey where grey squirrel culling over a seven year period reduced seroprevalance to 4% (Schuchert et al., 2014). The grey squirrel population surveyed here is a relatively stable, well established one, with no historical or current culling, which would explain the high levels of prevalence observed, that are probably reflective of the national UK population.

At the individual level, we found SQPV infections to be chronic in ˜80% of grey squirrels, but with SQPV infection histories in some being indicative of frequent viral recrudescence or reinfection, which is atypical in poxviruses (Boldogh et al., 1996). Immune protection against pox viruses is complex; defence from primary infection relies on the innate immune system, and in mice, the complement system is important to enable survival from mouse pox (ectromelia) exposure (Moulton et al., 2008), while antibodies appear to be important in the recovery of subsequent pox infections (Panchanathan et al., 2006). Notably, we found no link between antibody levels (serological titre) and presence or absence of the virus, but did find those with high viral presence at the forearm site had increasing serological titres. This suggests that both the antibody response is ineffective at controlling the virus, and that the forearm site is the main area of shedding and does not represent simple contamination of the area. Considering this, and the multiple peaks in serological response and viral shedding, it is not clear whether individual squirrels are able to clear infections but become reinfected, or whether infections remain at low levels, with bouts of recrudescence. While latency has not been described in poxviral diseases before, reinfection with viraemia is also a poorly documented finding. A second pox infection has been recorded in seropositive rabbits with myxoma virus (Marchandeau et al., 2014) and in orf virus in sheep (Haig and McInnes, 2002). Repeated smallpox infection has also been recorded in either previously infected or vaccinated people, though the level of morbidity was greatly reduced (Fenner et al., 1988). In addition, in some breeds of mice, ectromelia has been associated with subclinical shedding (Whary, 2015). Hence, more detailed observations are necessary to clarify the pathogenesis of these endemic poxviral infections in their native hosts.

As well as potentially affecting the presence or level of viral infection, the immunological status of the individual squirrel is also likely to have a large influence on levels of shedding of the poxvirus from infected animals. The grey squirrel's antebrachium (forearm) has been highlighted by earlier work (Atkin et al., 2010) as a site of potential SQPV shedding; the antebrachial area contains sebaceous glands responsible for scent secretion (as well as tactile vibrissae), so this could represent a possible route for SQPV distribution through scent marking and countermarking. We found increasing serological titre, reflecting increasing serocompetence, occurring in parallel with the quantitative level of SQPV shedding from the arm. This is the first time an association has been identified between serological analysis and viral load by PCR, and indicates that arm shedding of SQPV, in some individuals, can be a chronic stage (sometimes extending several months), despite the host having generated antibodies in response.

A key recurring factor in our analyses was the association of either prior or current ADV infection with various measures of SPQV infection. For example, we found a strong positive association between ADV

Table 2

Mixed model analysis with SQPV viraemic load among SQPV-positive animals from blood. Structure and abbreviations as described in Table 1. In addition, AD-V.past = ADV infection status (positive/negative) at the previous capture; Flea.past = flea presence/absence at the previous capture; Flea.future = flea presence/absence at the subsequent capture. Note that, for some lower rows in (a), reduced data sets had to be used because not all data points may have been available (e.g., for longitudinal analyses requiring repeat capture data from the same animal over successive sampling dates). In these cases the ΔAIC values quoted refer to the model in the row above, but with an AIC value for the reduced data set.

(a)

investigating factor	model; SQPV ~ (1|SQ ID) + ...	n(obs)	n(t)	AIC	ΔAIC
base model	month	120	52	414.08	–
wound	month + wound	107	49	373.72	−4.57
ADVp	month + wound + ADVp	107	49	365.73	−7.99
ADV.past	month + wound + ADVp + ADV.past	86	38	283.71	−3.55
Flea.past	month + wound + ADVp + ADV.past + flea.past	86	38	277.97	−9.29
Flea.future	month + wound + ADVp + ADV.past + flea.past + flea.future	85	38	277.20	−4.90

(b)

SQPV ~ (1\|SQ ID) + month + wound + ADVp + ADV.past + flea.past + flea.future

| Factor	AIC	ΔAIC	Coefficient	SE	t-value
(intercept)	272.74		−8.566	0.466	−18.384
Month:	282.58	9.85			
February			0.185	0.514	0.360
April			0.733	0.533	1.374
May			−1.225	0.731	−1.675
June			−0.710	0.851	−0.834
July			−0.734	0.842	−0.871
September			−0.224	0.659	−0.341
October			1.020	0.493	2.069
November			−0.048	0.514	−0.093
December			−0.482	0.544	−0.885
ADV (positive)	284.23	11.49	0.864	0.226	3.822
ADV.past (positive)	274.87	2.13	0.496	0.241	2.057
Wound (positive)	283.41	10.68	1.162	0.287	3.697
Flea.past (positive)	279.89	7.15	−0.738	0.238	−3.108
Flea.future (positive)	277.97	5.23	0.646	0.235	−2.747
SQ ID	270.74	−2			
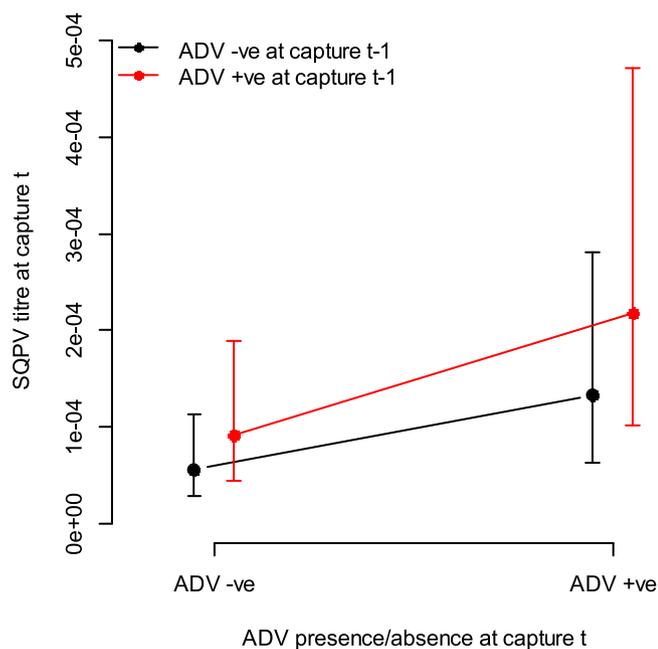
Fig. 2. Association between contemporary (at capture 't') and previous (at capture 't-1′) ADV infection status (positive or negative) on SQPx viral titre, based on qPCR of blood samples.

presence and contemporary SQPV presence in the blood, although the underlying mechanism of this relationship is uncertain. Since there was no detectable lagged effect (i.e., prior ADV infection leading to subsequent SQPV infection), this may suggest the epidemiologies of these two viruses, leading to viraemia, share common factors which would account for their coinciding occurrence (Fenton et al., 2014). For example, if the route of infection is similar for both viruses (e.g., faecal-oral), the acute phase of infection for SQPV or ADV will appear to be concurrent. Furthermore, the seasonal tendency for increased autumn/winter infection for both viruses may reflect a common combination of lack of food resources, peak juvenile population and colder temperatures. However, both the SQPV and ADV genomes encode several different immunomodulatory proteins which are capable of suppressing host immune function (Barthold et al., 2011; Darby et al., 2014). As such, the positive association of both infections could arise through a facilitatory interaction, via suppression of the host's immune system. This possibility is supported by our finding of a lagged association between the presence of ADV and the quantitative level of both current and subsequent SQPx viraemia. Hence, although prior ADV infection doesn't seem to affect subsequent susceptibility to (presence of) SQPV infection, it does seem to potentiate the magnitude of viraemia among those that get infected. The immunomodulatory roles of both SQPV (Darby et al., 2014) and ADV are established, so there are potentially several mechanisms whereby viral coinfection could lead to more severe pathogenic effects, due to changes in the quantitative viraemic load of the co-circulating viruses. Importantly, these subtle, but potentially powerful quantitative effects would be overlooked by studies

Table 3

Mixed model analysis with presence or absence of SQPV viraemia from arm swabs as the dependent variable. Structure and abbreviations as described in Tables 1 and 2. In addition, Δwt.future = change in body weight from current to subsequent trapping session; Δwt.past = change in body weight from previous to current trapping session; flea-count = absolute number of fleas observed at the current capture; osqpv_p = SQPV presence of viraemia from oral swab samples at the current capture.

(a)

investigating factor	model; asqpv_p ˜ (1|SQ ID) + ...	n(obs)	n(t)	AIC	ΔAIC
base model	Season	248	29	269.66	–
Δwt.past	Season + Δwt.past	243	29	211.45	**−2.46**
flea.count	Season + Δwt.past + flea.count	218	29	219.13	**−3.09**
osqpv_p	Season + Δwt.past + flea.count + osqpv_p	214	29	207.81	**−8.77**

(b)

	asqpv ˜ (1|SQ ID) + season + Δwt.past + flea.count + osqpv_p				
Factor	AIC	ΔAIC	Coefficient	SE	z-value
(intercept)	208.68	–	−0.404	0.363	−1.11
Season:	215.09	6.41			
Winter			1.949	0.681	2.86
Spring			0.377	0.427	0.86
Summer			−0.123	0.511	−0.24
Δwt.past	210.93	2.26	102.2	51.47	1.99
flea.count	211.05	2.37	0.165	0.086	1.92
osqpv_p	218.00	9.33	1.251	0.377	3.32
SQ ID	206.73	−1.95			

that only characterise infections as binary presence/absence variables.

As demonstrated by individual infection profiles, we propose that the arm shedding period is a chronic stage of SQPV infection, mainly occurring in the winter. In this context, it is to be expected that the effect of adenoviraemia (acute phase), either current or lagged, would have no significant effect on chronic SQPV presence from arm swabs. However, we detected a negative statistical effect of prior adenoviraemia on subsequent SQPV shedding in arm swab samples. The mechanism underlying this association is not currently known, but it may arise from SQPx viraemia and adenoviraemia being acute, whereas SQPV shedding is a chronic phase of the infection. Hence, adenoviraemia may potentially prolong the SQPV acute phase, so extending the time between the acute SQPV phase and the recovering chronic phase, so reducing viral shedding over the time scales examined. Further work, including finer-scale temporal sampling, would be needed to further elucidate the underlying mechanistic relationship between ADV

infection and SQPV shedding in co-infected animals.

Our results may clarify potential routes of SQPV transmission among grey squirrels. Previous studies failed to find any significant seasonality to SQPV infection in grey squirrels (Sainsbury et al., 2000, Bruemmer et al., 2010, Collins et al., 2014), which is unsurprising given their opportunistic data from culls and the fluctuating chronic infection shown here. Similarly, earlier studies had also discounted the possibility of ectoparasites acting as sole vectors (Sainsbury et al., 2000). The association of SQPV infection here during the winter would not traditionally correlate with the expected activity of ectoparasites, which peak in late summer, but it is of note that fleas were detected throughout all seasons. A modelling study has also supported the role of the flea as potential vectors in SQPV transmission (Cowan et al., 2016). While the analysis here has indicated associations with flea number and both SQPV infection in the blood and viral shedding from the arm, given the relatively low viral load of the blood compared to the relative

Table 4

Mixed model analysis with SQPV viraemic load among SQPV-positive animals from arm swab samples. Structure and abbreviations as described in Tables 1–3. In addition, ΔOD.past = change in Optical Density from ELISA of SQPV antibodies, from previous to current capture; aSQPV.past = SQPV load from arm swab samples at the previous capture.

(a)

investigating factor	model; aSQPV ˜ (1|SQ ID) + ...	n(obs)	n(t)	AIC	ΔAIC
base model	season	179	29	698.19	–
ΔOD.past	season + ΔOD.past	160	29	607.64	**−20.90**
ADV.past	season + ΔOD.past + ADV.past	157	29	587.31	**−5.97**
aSQPV.past	season + ΔOD.past + ADV.past + aSQPV.past	157	29	583.88	**−3.43**

(a)

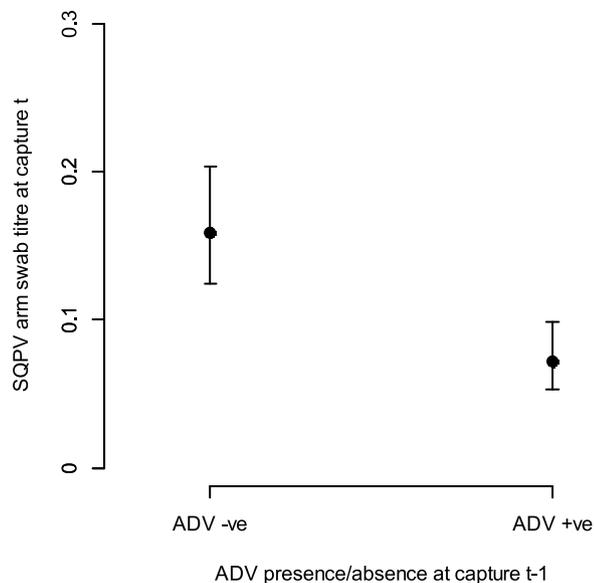|	aSQPV ˜ (1|SQ ID) + season + ΔOD.past + ADV.past + aSQPV.past				
Factor	AIC	ΔAIC	Coefficient	SE	t-value
(intercept)	583.88		−1.829	0.239	−7.642
Season:	596.96	13.08			
Winter			1.026	0.312	3.287
Spring			−0.303	0.324	−0.933
Summer			0.336	0.432	0.777
ΔOD.past	606.01	22.13	19.768	3.871	5.107
ADV.past (positive)	590.52	6.64	−0.791	0.265	−2.980
aSQPV.past (positive)	587.31	3.43	0.100	0.043	2.349
SQ ID	581.88	−2			

Fig. 3. Association between ADV infection status (positive or negative) at the previous capture ('t-1') and SQPV load at the subsequent capture ('t'), based on qPCR of arm swab samples.

quantity found around the arm (*c.* 250 times more), and the proposed short viraemic period, we suggest flea vectoring likely represents a minor transmission route. Given the higher magnitude of infection found in the forearm, it is logical to suggest (taking account of a squirrel's manual feeding) that contact spread of SQPV, during feeding or scent marking, may play an important role in viral transmission. ADV is thought to be transmitted by the faeco-oral route (probably following a viraemia), thus representing subacute infection, as reported here. The coinfection link between the two viruses further supports faeco-oral transmission of SQPV occurring during feeding.

This study provided a rare insight into the dynamics of SQPV in wild populations of grey squirrels. The densities and demographics of the population are compatible with those reported elsewhere (Bruemmer et al., 2010; Sainsbury et al., 2000) so there is no reason to suggest this population would not be representative of the wider national grey squirrel population. By understanding the dynamics of such a virus of conservation concern in its reservoir host population, it may be possible to develop better predictive models of the timing and factors that lead to increased risk to the target host species (Fenton and Pedersen, 2005; Lloyd-Smith et al., 2009; Viana et al., 2014), in this case red squirrels. The predictions made from such models are often highly sensitive to estimated parameters and necessary statistical assumptions; hence the data reported here should help in developing more robust models with valid assumptions and parameters. For instance this study would suggest that a simple SIR (susceptible, infected and recovered) model would not be suitable for simulating SQPV infection in grey squirrels given the chronic viral shedding with no apparent recovery identified here, and possible recrudescence or reinfection over prolonged periods of time. More broadly, our findings show the value of fine resolution, longitudinal studies that use quantitative measures of infection and shedding, to clarify drivers of pathogen dynamics within wildlife reservoir populations.

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Designed the study: TD MB JC. Fieldwork: TD JC DJ.
Sample processing: TD DJ. Molecular analysis: TD DJ. Statistical analysis: TD AF.
Discussed results and wrote the manuscript: AF TD JC MB.
All authors approved the final version of the manuscript.

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