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Research Article

Introduced Canadian Eastern grey squirrels: squirrelpox virus surveillance and why nothing matters

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Abstract

Squirrelpox virus (SQPV), an unapparent infection of the Eastern grey squirrel (*Sciurus carolinensis*), is considered to be mediating the ecological replacement of the Eurasian red squirrel (*Sciurus vulgaris*) in the United Kingdom (UK) and Ireland. Evidence suggests that the Eastern grey squirrel is the natural reservoir host of SQPV and therefore there is considerable concern amongst conservationists that when translocated out of its natural range in North America, the Eastern grey squirrel could pose a similar threat to encountered indigenous squirrel populations. Serum samples collected from Eastern grey squirrels from British Columbia (BC), Canada, an introduced population derived from squirrels translocated at the beginning of the 20th Century, were surveyed for evidence of antibodies against SQPV. None of the 130 samples tested had antibodies to the virus, contrasting with 15 out of 19 (79%) positive serum samples collected from Ontario, a population within the natural range of the Eastern grey squirrel. We conclude that BC is currently free of SQPV and that to maintain the virus-free status further translocations of grey squirrels from their native range should be prevented. A comprehensive study of the founding population and comparison with the other populations in BC, including broad epidemiological surveillance for the virus is recommended as an early warning for potential incursions of the virus and the threats that this may pose.

Introduction

The Eastern grey squirrel (*Sciurus carolinensis*) is recognised as an invasive non-native species (INNS), also referred to as an Invasive Alien Species (IAS), wherever it has been introduced outside of its historic natural range in the eastern hardwood forests of North America (Bertolino 2009). To date, it has been introduced to Australia, South Africa, Italy, Ireland and the United Kingdom (UK) (Lawton et al., 2010; Gurnell, 1987; Davis, 1950), and also to locations in North America beyond its native range (Koprowski, 1994). Although the population in Australia was eradicated (Peacock, 2009), grey squirrels continue to affect native flora and fauna in the UK, Ireland, Italy and South Africa. This is most notably so in the UK and Ireland (Gurnell et al., 2015; Sainsbury et al., 2008) where the grey squirrels have substantially displaced the indigenous Eurasian red squirrel (*Sciurus vulgaris*).

Grey squirrels were introduced from the USA to the UK in the late 19th century and translocated from there to Ireland in the early 20th century (Middleton, 1930, 1931). Although it was quickly documented that native red squirrels were disappearing from many parts of England and Wales and being replaced by grey squirrels, the drivers for this process were not fully understood (Shorten, 1954). It was many decades later that it was realised that the ecological replacement of the red squirrels was being driven by resource competition (Gurnell et al., 2004) and via epizootic disease caused by Squirrelpox virus (SQPV). The disease, squirrelpox, is invariably fatal for the red squirrels but has little discernible effect on grey squirrels (Fiegna, 2011; Fiegna et al., 2016; Atkin et al., 2010; Rushton et al., 2006; Tompkins et al., 2002; Sainsbury et al., 2000; Sainsbury and Ward, 1996). Models predicted that although grey squirrels were likely to be able to displace red squirrels through more efficient use of woodland resources, the displacement would happen up to 25 times faster in the presence of squirrelpox (Rushton et al., 2006). In Ireland, the situation appears to be identical although there, it took longer to confirm the devastating effects of the virus (McInnes et al., 2013).

In Italy, grey squirrels were also introduced from the USA, but not until the 1940s (Bertolino et al., 2013). Models simulating range expansion in the absence of control measures, projected the northern Italian populations could expand into neighbouring countries within 60 years (Bertolino et al., 2008), potentially threatening the wider continental population of the Eurasian red squirrel. Recent studies within Italy have shown the complexity of the interaction between the introduced grey squirrels and the native red species, including their influence on personality within red squirrels, with higher expressions of sociability in areas where they are sympatric with grey squirrels compared to red-only areas (Wauters et al., 2019). This in turn may contribute to the spillover of infections from one species to the other. It has already been shown that the macroparasite (*Strongyloides robustus*) has been passed from grey to red squirrels disrupting the natural host-parasite interactions in the native species and reducing the amount

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of costly behavioural traits such as activity (Santicchia et al., 2020). This in part may help the grey squirrel to outcompete the red species. However, another important finding is that the presence of sympatric grey squirrels has been shown to increase stress in red squirrels, potentially making them more susceptible to infections such as with SQPV (Santicchia et al., 2018). To better understand the nature of the threat posed by the introduced grey squirrels, a recent study (Romeo et al., 2019) investigated whether squirrelpox infection could be detected in Italian grey squirrels. Fortunately, no evidence of the virus was found and thus it was concluded that ecological replacement of red squirrels by grey squirrels in Italy was caused exclusively by competition (Gurnell et al., 2004, 2015) and not being driven by SQPV as it is in the UK and Ireland.

The native range of *Sciurus carolinensis* is mainly in the eastern United States and adjacent provinces of Canada: southern Quebec, Ontario and Manitoba, then south to eastern Texas and Florida (Koprowski et al., 2016). However, various translocations of the species into other parts of North America have taken place; these include the westernmost states of the USA as well as the Canadian provinces of New Brunswick, Nova Scotia, Saskatchewan, Alberta and British Columbia (BC).

The original appearance of grey squirrels in BC is linked to a deliberate release of animals from New York into a municipal park (Stanley Park) in Vancouver in the early 1900s. Whether this population then served as a source for the relatively more recent establishments on Vancouver Island and the Okanagan Valley is presumed, but has never been fully established (Larsen, 2016). In BC, the grey squirrel is considered a potential threat to the endangered Garry Oak (*Quercus garryana*) ecosystem in the south-western corner of the province, but suspected impacts upon the indigenous North American red squirrel (*Tamiasciurus hudsonicus*) and Douglas squirrel (*T. douglasii*) remain poorly studied (Larsen, 2016; Gonzales, 2005; Bruemmer et al., 1999). Although conservationists have urged the control of grey squirrels, the window of opportunity over much of the province has been lost due to management inaction and the rapid expansion of the species (Larsen, 2016). One putative threat not yet investigated in BC is the potential for the introduced grey squirrels to be carrying SQPV.

This study set out to determine whether or not there is physiological evidence of the Eastern grey squirrels in BC carrying SQPV. Serological analysis was performed on blood samples collected from populations of introduced grey squirrels across the province (Fig. 1). The samples were collected at two time points separated by approximately 12 years and compared with blood samples taken from Eastern grey squirrels from Ontario. Squirrel carcasses were examined for evidence of skin disease and PCR analysis of hair samples for the virus was undertaken.

Methods

Serological analysis for antibodies against SQPV

Serum samples (Tab. 1) were opportunistically collected post-mortem from 120 grey squirrels being actively controlled or euthanized at wildlife/veterinary centres and/or killed in road traffic accidents. An additional 30 animals were sampled through live-trapping and euthanasia in 2016/17, yielding a further 29 serum samples. These additional 30 animals were under Animal Use Protocol 101548 issued to KL by the Thompson Rivers University Animal Ethics Committee, and British Columbia Wildlife Act permit MRKA17-26541. The presence in the serum of antibodies against SQPV was determined using the ELISA previously described by Sainsbury et al. (2000). Generally, blood samples were stored frozen at −20 °C as soon as possible after collection. These were processed through to serum and tested in the ELISA within 12 months of collection. Where blood could not easily be collected, due to the time of death or condition of the carcass, fluid from the body cavity was collected for testing.

A total of 19 samples were from Toronto (Ontario), a population of Eastern grey squirrels within the natural range of the species. Polymerase Chain Reaction (PCR) analysis for SQPV DNA.

Tail hair samples were available from 69 animals. Sixty of these 69 squirrels also yielded a serum sample that was tested in the SQPV ELISA (above). DNA was purified from the tail hair samples and PCR analysis was undertaken for SQPV DNA (30 hairs/sample) using the newly developed inner and outer primer and probe nested qPCR methodology as described elsewhere (Everest et al., 2019; Shuttleworth et al., 2019).

Figure 1 – Eastern grey squirrel distribution and sampling points in British Columbia, Canada. The extent of the known populations of the Eastern grey squirrels in British Columbia are indicated in black shading around Vancouver, on Vancouver Island and in the Okanagan Valley around Kelowna, where serum samples were primarily collected. Inset: Map showing North America and the natural range of the Eastern grey squirrel (in black shading), Vancouver Island is also indicated..

Negative stain Transmission Electron Microscopy (TEM)

TEM was undertaken on a single scab sample to determine if poxvirus particles could be detected as an indication of squirrelpox disease. The methodology was undertaken as described in (Everest et al., 2010).

Negative Predictive Value analysis

To establish the level of confidence in our results we calculated the herd-level negative predictive value (HNPV) of the applied diagnostic tests as described in Romeo et al. (2019). Based on sample size, test sensitivity and specificity, and expected prevalence, HNPV allows an estimation of the probability that a pathogen is truly absent from a sample given that a number of individuals all test negative for it (Christensen and Gardner, 2000). Based on literature data about SQPV circulation in infected grey squirrel populations (Collins et al., 2014; McInnes et al., 2013; Sainsbury et al., 2000), we simulated two different scenarios, assuming an expected infection prevalence of either 25 or 50%. ELISA sensitivity and specificity were set at 90% and 92%, respectively (Sainsbury et al., 2000), and qPCR at 99% for both sensitivity and specificity.

Results and Discussion

Serology

The SQPV antibody ELISA was performed using serum samples taken from animals in BC over a period of 12 years. Originally, in 2005 sampling was centred around Vancouver and Vancouver Island with 75 samples being collected. None tested positive for antibodies against SQPV. A further 55 serum samples were collected between 2014 – 17, again from Vancouver Island, but also from Kelowna, BC, approximately 400 kilometres east of Vancouver, on mainland Canada, where the Eastern grey squirrels had been established since 2012 (Larsen, 2016). Again, none of the samples tested positive for antibodies against SQPV. This contrasts with 15 out of 19 (79%) samples, positive for antibodies against SQPV, which had been collected from free-ranging grey squirrels in the grounds of Toronto Zoo (Ontario) over the winter months of 2000 – 2001 (Tab. 2).

PCR and electron microscopy

Initially, two of the 69 tail hair samples tested for the presence of SQPV DNA recorded borderline single values (Ct scores of 27 and 30 respectively), from a duplicate aliquot sample, that were near the cut-off value for ascribing positive/negative results. This value was determined from

Table 1 – Origin and dates of Samples.

Approximate Geographical origin	No. of Samples	Approximate Date of sampling
Ontario		
Toronto Zoo (free-ranging)	9	Oct/Nov 2000
Toronto Zoo (free-ranging)	10	Jan/Feb 2001
Lower Mainland British Columbia		
Vancouver City	1	Jun 2017
Vancouver Island British Columbia		
Vancouver Island	32	May-Jul 2005
Duncan	3	Jul 2005
Nanaimo	13	Winter 2016
Nanaimo	6	Mar-Dec 2017
Victoria	14	May 2005
Victoria	26	Jul 2005
Victoria	10	Winter 2016
Kelowna British Columbia		
Kelowna	3	Mar/Dec (2) 2014
Kelowna	23	May/June 2017

a serial set of dilutions assayed in triplicate from a positive PCR sample, which had previously been determined as SQPV particle positive by TEM and confirmed by sequencing to be SQPV. The dilutions were analysed and a Ct value of 27 was determined as the lowest value to reliably produce a positive result. Both samples that had initially given borderline positive results were repeated, but no amplicon was recorded in either of the repeated duplicate sample wells and thus the samples were re-classified as negative.

Generally, no lesions indicative of a squirrelpox lesion were observed on any of the carcasses examined. One small hind leg scab, from a grey squirrel from Kelowna, was examined by negative stain TEM, but no viral particles were observed.

Negative Predictive Value

Assuming that SQPV had an actual prevalence in our sampled population of either 50 or 25%, the estimated herd-level negative predictive value for both diagnostic tests were 100% and 99.9%, respectively. This means that in both scenarios, given that all of the sampled individuals tested negative in the ELISA and qPCR assay, the probability that the examined population is negative for exposure to SQPV is higher than 99.9%.

Discussion

Our findings reaffirm the importance of surveillance for the presence of SQPV to be conducted amongst discrete regional populations of Eastern grey squirrels, particularly where they are present outside of the species' natural range. In BC, we concentrated on the established populations of the Eastern grey squirrel in and around Vancouver and the Okanagan Valley, although other less established, and consequently less visible, populations may exist elsewhere in the province particularly since the occasional anthropogenic translocation of the species is suspected (Larsen, 2016). The study failed to find antibodies against SQPV within any of main grey squirrel populations introduced to BC, whereas, in contrast, antibodies against the virus were present within Eastern grey squirrels sampled from Toronto, a population within the native Canadian range. Although failure to detect any signs of squirrelpox disease, or any antibodies against the virus, does not confirm absence of SQPV from BC, the estimation of HNPV indicates that the probability that the sampled population is truly negative is higher than 99.9%. It is impossible to know what proportion of the grey squirrel population was tested from each of the locations in BC, but previous studies with SQPV and other poxviruses in the UK and Ireland would suggest that if the virus is present, it would be readily detectable by serology (Collins et al., 2014; McInnes et al., 2013; Sainsbury et al., 2000). For example, previous investigations on the prevalence of Cowpoxvirus, another poxvirus where disease signs are absent or not immediately obvious in the reservoir host, suggested that if the virus was circulating within a population of its reservoir host, upwards of 20% of that population would be expected to be seropositive (Crouch et al., 1995). In the UK, the proportion of SQPV-seropositive grey squirrels in a number of woodlands, where the population was well established, was in excess of 80% (Sainsbury et al., 2000), very similar to what was found with the grey squirrels from their natural range in Toronto. In Ireland, in contrast, in certain woodlands where it was considered that infected grey squirrels were emerging for the first time, it was noted that the proportion of grey squirrels that tested seropositive for antibodies to SQPV steadily rose over a 10 – 12 year period from an average of 17% being seropositive to an average of 67% a decade later (McInnes et al., 2013). Thus the fact that the samples in our study were collected, in particular from Vancouver Island, over a twelve year period may have increased our chances of detecting SQPV if it were present in any of the populations studied.

In Europe, when introduced grey squirrels carry the infection, SQPV is associated with epidemic disease in sympatric native Eurasian red squirrel populations (McInnes et al., 2015). This is certainly true in the British Isles. However, no SQPV-associated disease has been found on the European mainland and a recent study by Romeo et al. (2019) indicated that the virus was likely to be absent from the introduced mainland European (Italian) populations. The authors postulated that either SQPV had been absent from the Italian founders, or that if the infection was originally present it was lost during the population establishment phase, possibly due to there not being sufficient grey squirrel numbers to sustain it. The small number of original founders to western Canada may similarly explain the absence of squirrelpox observed today. In BC, grey squirrels were first introduced to Stanley Park in 1909 (Nagorsen, 2005) long before any rules on the introduction of alien species and it is likely that both of the more recent population establishments in Kelowna and on Vancouver Island were based on founders obtained from Stanley Park rather than from separate translocations from eastern Canada (Larsen, 2016). The current legislation surrounding introductions of alien species, as well as the possession of captive species as pets, varies tremendously over the North American continent. In BC, transport or possession of captive grey squirrels is not permissible, although the rehabilitation and subsequent release of the animals is a more complicated issue with mixed enforcement. Nevertheless, so successful has been the establishment of the grey squirrel in BC that the significant populations that are now present mean that should an SQPV-infected animal be translocated, either intentionally or unintentionally from the eastern Canadian populations, there is a very real risk that the infection would be able to persist.

The North American red squirrel is now regionally sympatric with the Eastern grey squirrel within both eastern and western Canada. A poxvirus-associated disease in this species has been reported previously. In 2009, a single North American red squirrel *Tamiasciurus hudsonicus* from the Yukon territories was found having succumbed to pox-like lesions. Upon investigation, however, the infection was determined to have been caused by an orthopoxvirus, completely separate from the SQPV responsible for deaths in the Eurasian red squirrel (Himsworth et al., 2013). The origins and natural host of the or-

Table 2 – Serological screening results for SQPV.

Approximate Geographical origin	No. of Samples ELISA positive/Total tested	Approximate Date of sampling
Toronto Zoo, Ontario	15/19	2000/2001
Vancouver Island B.C (greys present since 1960s)	0/32	2005
Duncan	0/3	2005
Nanaimo	0/19	2016/2017
Victoria	0/40	2005
Victoria	0/10	2016
Kelowna (greys present since early 2000s)	0/3	2014
Kelowna	0/23	2017

thopoxvirus, and its epidemiology with respect to the North American red squirrels or indeed the grey squirrels, remain obscure.

SQPV epidemiology in the native red squirrel also remains undefined, and to date there is no evidence of the infection being associated with disease let alone producing epidemic mortality. This may be fortuitous or as a result of the red and grey species co-evolving over many years in the same landscape, being exposed to the same pathogens. In contrast, the Douglas squirrel is native only to western North America. In BC, populations persist with sympatric introduced grey squirrels e.g. the lower mainland of the province (Gonzales, 2005) suggesting that inter-specific competition is either absent or at a low level. Unlike the North American red squirrel, this native species did not evolve with the Eastern grey squirrel and, historically, is therefore unlikely to have encountered SQPV making it potentially more akin to the naïve populations of the Eurasian red squirrels that are highly susceptible to disease. The absence of SQPV in grey squirrels within BC must therefore be considered fortunate and again reinforces the need to prevent translocation of grey squirrels from eastern Canada.

Populations of wild squirrels carry many infections (Romeo et al., 2016; Greenwood and Sanchez, 2002) and co-infections can exacerbate or change the epidemiology of endemic disease, potentially increasing the threat to sympatric animal populations (Chantrey et al., 2019). It is clear that not all the translocations of Eastern grey squirrels have taken SQPV with them as evidenced by the Italian grey squirrels, some of the original UK founder populations, and this study. It is considered unlikely that this is because there is a subspecies or sub population of the Eastern grey squirrel that is genetically more resistant to the virus than others, but a better understanding of the epidemiology of SQPV infection in the Eastern grey squirrel in its native range is required. Indeed, the presence of the virus in the Eastern grey squirrel can be considered cryptic as its discovery only came with its emergence in a susceptible sympatric population of red squirrels in Europe and nothing is currently known about its prevalence across North America. A more comprehensive study of the founding population of grey squirrels in Stanley Park and its relationship to the other BC populations, those other invasive populations in western Canada and beyond, and Eastern grey squirrels from the native range is merited. This, along with wider epidemiological surveillance for SQPV, including in the potentially susceptible indigenous North American red squirrels, will help our understanding of SQPV in its natural habitat and may help answer the question as to whether or not the invasive grey squirrels in BC pose a disease threat to the indigenous red squirrel species as they do in the UK.

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