Disease, invasions and conservation: No evidence of squirrelpox virus in grey squirrels introduced to Italy

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Short Title No squirrelpox virus in Italian grey squirrels

Abstract

Native red squirrels (*Sciurus vulgaris*) in Great Britain and Ireland are threatened by alien grey squirrels (*S. carolinensis*) through exploitation competition and spillover of squirrelpox virus (SQPV). By accelerating the replacement of red squirrels by the invader, SQPV represents a fundamental factor to consider when planning management and conservation strategies. In mainland Europe, grey squirrels introduced to Italy threaten the survival of the whole continental red squirrel population, but no extensive surveys for SQPV presence have been carried out in the region. We therefore investigated SQPV infection in north Italian grey squirrel populations through a combination of serological and molecular methods. Firstly, we analysed sera from 285 individuals through an enzyme-linked immunosorbent assay (ELISA) to detect antibodies against SQPV. Secondly, a PCR designed to amplify a segment of the G8R SQPV gene was carried out on DNA extracted from swabs and skin tissue samples from a second set of 66 grey squirrels. ELISA tests identified 4 reactors (1.4%), but the subsequent PCR survey did not detect any SQPV DNA. Based on the low prevalence observed and on PCR results, we believe that the 4 suspected positives were the result of an ELISA cross-reaction following exposure to another pox virus. Considering sample size and performances of the two methods, confidence of freedom from SQPV resulted above 99.9%. However, because of the severe impact of SQPV on red squirrels, we recommend the implementation of a passive surveillance plan for the early detection of an SQPV emergence in continental Europe.

Keywords

Sciurus vulgaris; Sciurus carolinensis; spillover; SQPV; invasive alien species; disease-mediated invasions

Introduction

During the last century, populations of Eurasian red squirrels (*Sciurus vulgaris*) declined throughout Great Britain and large areas of Ireland due to habitat destruction and fragmentation and interspecific competition with introduced Eastern grey squirrels (*Sciurus carolinensis*) (Gurnell, Lurz & Wauters, 2015). Replacement of native red by alien grey squirrels is one of the best documented examples of the negative impact induced by biological invasions on native ecosystems, and is mainly based on an exploitation competition. Grey squirrels make a better use of food resources than the native species, leading to a progressive reduction in the fitness of red squirrels, which over time will result in the local extinction of red squirrel populations (Gurnell *et al.*, 2004; Wauters, Tosi & Gurnell, 2005). Additionally, the interaction between the two species in Great Britain and Ireland is also mediated by a shared pathogen, which accelerates the replacement of the native rodent by the invader: the squirrelpox virus (SQPV).

The SQPV-red-grey squirrel system is an emblematic case that highlights the importance of infectious diseases in biological invasions and, more in general, in biodiversity conservation (Daszak, Cunningham & Hyatt, 2000; Dunn *et al.*, 2012). In general, biological invasions represent a perfect scenario for disease-mediated interactions to occur because it is likely that invaders will carry along alien pathogens that may spill over to native species (Hudson & Greenman, 1998; Strauss, White & Boots, 2012) or they can amplify local parasites circulation, leading to spill-back processes towards native hosts (Kelly *et al.*, 2009; Strauss *et al.*, 2012). In particular, when spillover occurs, it is also likely that the impact of the shared pathogen will be highly asymmetrical, as invaders will successfully introduce only pathogens that are relatively avirulent to them, but that may have a severe impact on native hosts which lack any previous exposure to the disease (Strauss *et al*., 2012; Lymbery *et al.*, 2014). In recent years, the role played by alien species in disease emergence in wildlife has been increasingly recognised, and several examples of disease-mediated invasions involving both vertebrate and invertebrate hosts have been documented (e.g. Strauss *et*

al., 2012; Lymbery *et al.*, 2014; Tompkins *et al.* 2015 and references therein). Awareness about disease risks connected to invasions is thus growing and there is a compelling need to account for such threats in management and control strategies (e.g. Dunn & Hatcher, 2015).

The red-grey squirrel system in Great Britain is indeed a prominent example of disease-mediated invasions because SQPV has a very different pathogenicity in the two hosts, with grey squirrels seemingly unaffected by the infection and high mortality rates in red squirrels (Tompkins *et al.*, 2002; Fiegna *et al.* 2016). As a result, the virus ultimately facilitates replacement of the highly vulnerable native species by the more tolerant invader (Tompkins *et al.*, 2002; Tompkins, White & Boots, 2003). It has been long debated whether the pox infection was introduced in Great Britain and Ireland by the alien host or was already endemic in the area, but most evidence points toward the former hypothesis (McInnes *et al.*, 2006). In any case, regardless of SQPV origins, it is now certain that grey squirrels act as a reservoir for the virus, maintaining its circulation with dire consequences for native red squirrels (Sainsbury *et al.*, 2000; Chantrey *et al.*, 2014). Even before the role played by SQPV was recognised, early simulations based on exploitation competition alone failed to explain the rate of red squirrel decline observed in Great Britain (Rushton *et al*., 1997). In the early 2000s, Tompkins and colleagues demonstrated firstly that SQPV has a differential impact on the two squirrel species (Tompkins *et al.*, 2002) and subsequently, by incorporating the disease in modelling analyses, that the virus accelerates replacement of red squirrels by grey squirrels (Tompkins *et al.*, 2003). Where SQPV is present, the grey squirrel replaces red squirrels up to 25 times faster than in areas without the infection, where only competition for resources occurs (Rushton *et al.*, 2006). SQPV in the UK appears thus as a crucial driver in the interaction between the alien and the native species and collecting data on its presence is fundamental in order to plan adequate management and conservation strategies (Gurnell *et al.*, 2006; Schuchert *et al.*, 2014; Macpherson *et al.*, 2015; Bertolino *et al.* 2016; White *et al.*, 2016).

In Europe, in addition to Great Britain and Ireland, grey squirrels have been introduced into Italy where their expansion could potentially threaten the whole continental red squirrel population

(Bertolino *et al.*, 2014). Distribution of the alien species in the country is still fragmented, with two large, expanding populations in Piedmont and Lombardy regions in north-western Italy and a smaller one in central Italy (Umbria region) (Martinoli *et al.*, 2010; Bertolino *et al.*, 2014; Signorile, Paoloni & Reuman, 2014a). Additionally, a small, isolated nucleus inhabiting an urban park in Genova Nervi (Liguria) is being eradicated through sterilization; and occasional sightings have been reported in Tuscany, Lazio and Veneto regions (Mori *et al.*, 2016). The two north Italian populations are located 100 km apart, near the borders with France and Switzerland, and include approximately 40,000 individuals which represent 90%-95% of the Italian grey squirrels. Expansion models predicted that, in absence of control, grey squirrels inhabiting these regions will cross the Alps and invade neighbouring countries within 60 years (Tattoni *et al.*, 2006; Bertolino *et al.*, 2008). The smaller population established in central Italy has a more recent origin and currently covers a 50 km^2 area around the town of Perugia, 350 km south of the northern nuclei (Signorile *et al.*, 2014a; La Morgia *et al.*, 2017). It is known that the large population in Piedmont was founded by a first, single introduction of four American squirrels in 1948 (Bertolino, 2009; Martinoli *et al.*, 2010), and genetic profiling suggests that the whole population in Perugia and at least some of the Lombardy nuclei derived from within-country translocations of Piedmontese individuals (Signorile *et al.*, 2016).

Red squirrels are widespread in most of the Italian peninsula except for heavily urbanized areas, the islands and the southernmost regions, with the presence of an endemic subspecies (*S. v. italicus*) in central Italy. Additionally, a new, endemic squirrel species (*S. meridionalis*) inhabiting Basilicata and Calabria regions in the south has been recently described (Wauters *et al.*, 2017). Threats to red squirrel survival in Italy include habitat loss and fragmentation, and direct competition with grey squirrels (Wauters *et al.*, 2002a, 2005; Wauters, Tosi & Gurnell, 2002b), with the local extinction of red squirrels from large areas where the invader is spreading (Bertolino *et al.*, 2014). However, the rate of grey squirrel spread (and concurrently of red squirrel decline) observed in the country always appeared much lower than in Great Britain (Bertolino *et al.* 2014). Absence of SQPV

infection, higher habitat fragmentation, reduced propagule pressure and genetic diversity have all been among the proposed mechanisms to explain the slower expansion of grey squirrels in Italy (Lurz *et al.*, 2001; Rushton *et al.*, 2006; Signorile et al, 2014b). All the three main Italian grey squirrel populations are currently under intensive control to prevent further expansion, but no surveillance for SQPV has been ever carried out in the country. Based on the British experience, the presence of SQPV in the Italian scenario could accelerate grey squirrel spread and potentially have huge welfare and conservation implications for the continental red squirrel population and for the survival of the two abovementioned endemic Italian taxa, *S. v. italicus* and *S. meridionalis*. To date, no diseased red squirrels with clinical symptoms of the infection have ever been reported in Italy, but this lack of evidence is not a proof of SQPV absence. Because of the often cryptic nature of the native squirrel, such disease may indeed have gone undetected, as was the case in Ireland for many years. There, it was known from enzyme-linked immunosorbent assay (ELISA) results obtained in the 90's that grey squirrels had been exposed to the virus, but it was not until 14 years later that disease was confirmed in red squirrels (McInnes *et al.*, 2013; Stritch *et al.*, 2015).

For this reason, and because of the conservation implications that SQPV presence in Italy could have, here we aim to address the lack of data on the prevalence of SQPV in Italian grey squirrels. Based on the apparent lack of diseased red squirrels and the relatively slow rates of species replacement observed in the country, we predict that Italian squirrel populations are free from SQPV infection. We will investigate this hypothesis by using a combination of serological and molecular testing, integrated with an analytical approach to estimate the likelihood of true absence of the infection.

Materials and Methods

Host-virus system

Infection by SQPV in grey squirrels is mostly sub-clinical (Tompkins *et al.*, 2002; Atkin *et al.*, 2010), with seroprevalence in infected populations reaching values from 25% up to 100% (Sainsbury *et al.*, 2000; Bruemmer *et al.*, 2010; Chantrey *et al.*, 2014; Collins *et al.*, 2014). In contrast, in red squirrels the virus causes skin lesions and severe exudative dermatitis on the face, feet and genitalia, leading to the death of infected individuals in a few weeks (Tompkins *et al.*, 2002; Carroll *et al.*, 2009; Fiegna *et al.*, 2016). Current evidence suggests that interspecific transmission does not require direct contact among individuals, since skin lesions are rich in viral particles that are thus likely to contaminate nests, branches or may even be carried by ectoparasitic vectors (Atkin *et al.*, 2010; Collins *et al.*, 2014; Cowan *et al.*, 2016; Fiegna *et al.*, 2016). It appears that some red squirrels are able to survive exposure to SQPV, but the infection generally causes high morbidity and mortality in the Eurasian species (Tompkins *et al.*, 2002; Sainsbury *et al.*, 2008; Shuttleworth *et al.*, 2015). Chantrey *et al.* (2014) estimated a population decline of approximately 90% with a potential survival rate of <10% following a naturally occurring epidemic of SQPV in red squirrels on Merseyside, UK.

Sampling and study sites

Between 2011 and 2014 extensive trapping of grey squirrels was carried out in the two main Italian populations located in north-western Italy. The Piedmont population covers approximately 2000 km^2 (Bertolino *et al.* 2014) with an estimated size of 25,000 individuals (min-max 15,600-45,800; LIFE09 NAT/IT/00095 EC-SQUARE Final Report, 2015). The Lombardy population is 100 km to the east and consists of several nuclei, more or less interconnected, for an estimated size of about 15,000 individuals (min-max 10,000-20,000; Bertolino & Wauters, unpublished data). To investigate SQPV presence, we collected samples from sixteen sites located in the two regions (Fig. 1) that were selected based on local squirrel density and to cover the maximum extent of the invader's known distribution in the two areas.

During 2011 and 2012 we carried out a first survey through serological testing, then (2013-2014) we carried out a second, separate sampling for SQPV detection through molecular methods. During both sampling campaigns, in each site we carried out a minimum of two trapping sessions that lasted at least 3 consecutive days. Squirrels were captured using live-traps (model 202, Tomahawk Live Trap Co., Wisconsin, USA) baited with hazelnuts that were checked at least twice a day (see Romeo *et al.*, 2014, 2015 for further details on trapping and handling methods). Captures were carried out mostly within an alien squirrels control program (LIFE09 NAT/IT/00095 EC-SQUARE): animals were immediately euthanised on the field by CO_2 inhalation and blood samples for serological testing were collected post-mortem through heart-puncture. In a few areas at the initial stages of the survey, the project was granted only permits for trapping and release. In this case, squirrels were marked with ear tags and released after blood collection from the femoral vein. Sampled blood was separated by centrifugation (15 min at 1800 g) within a few hours after collection and sera were subsequently stored at -20°C until analysis. Sampling for molecular analysis was carried out exclusively from culled animals: we collected swabs and skin tissue samples (approximate size 0.5 cm^2) from body areas known for having a predilection for SQPV infection (i.e. lip, eyelid, arm sensory vibrissae and flank from both sides of the body, Dale *et al.*, 2016). These were stored at -20°C until DNA extraction could be carried out. All the sampled individuals were visually inspected for lesions and the sample set was representative of the population structure for sex (167 females and 184 males) and age class (266 adults and 85 subadults). Trapping was carried out all through the year (176 individuals were trapped during spring-summer and 175 during autumn-winter).

Serological analyses

Grey squirrel serum samples were tested for the presence of antibodies against squirrelpox virus as previously described (Sainsbury *et al.*, 2000). Briefly, 285 sera were analysed against squirrelpox virus antigen, from cell culture-grown virus. ELISA plates (96-well flat-bottomed, Griener Bio-

One, UK) were coated with detergent-extracted (IGEPAL® CA-630; Sigma-Aldrich) antigen from SQPV-infected or mock-infected cells. Squirrel sera, diluted 1/50 in 1 x PBS / 0.05% v/v Tween 20/1% w/v bovine serum albumin, were added to duplicate wells containing SQPV or control negative antigen. After incubation for two hours, the wells were washed and bound IgG detected using Protein-G conjugated to horseradish peroxidise (HRP) and the substrate TMB (Sure Blu™ TMB Microwell Peroxidase substrate, KPL, USA). The optical density at 450nm was determined for positive and negative antigen wells and the corrected OD_{450} calculated for each serum sample. An OD_{450} value of >0.2 was considered positive.

Molecular analysis

Sixty-six individuals were examined for the presence of SQPV DNA through the analysis of swabs and skin samples previously collected from lips, eyelids, arm vibrissae and flanks. The swab samples were used as an initial survey and if any amplification was recorded in any of the reactions then the skin samples from that individual were subsequently analysed. DNA was isolated from samples using a commercially available kit (DNeasy® Blood and Tissue kit, Qiagen, Manchester, UK). For skin samples the manufacturer's recommended protocol was used on 25mg of tissue, while a modified protocol was used for swab samples (Dale *et al.*, 2016). DNA extracts were then stored at -20°C. Prior to analysis, the nucleic acid concentration of DNA extracts was measured using a NanoDrop 1000 (Nano Drop Technologies Inc., Wilmington, USA) and each diluted to a concentration of 20ng/µl. 'No sample' DNA extracts were run in tandem with each batch of extracts to act as potential contamination indicators. A quantitative multiplex PCR designed to amplify a segment of the grey squirrel phosphoglycerate kinase (PGK) gene (acting as an endogenous control) and a segment of the G8R SQPV gene were then used to analyse the DNA extracts. Cycling was carried out on a Lightcycler® 480 II (Roche, Wellwyn Garden City, UK) real-time PCR machine using the following primers and probes GGTCTATTATCCTGTTGGA (left PGK primer), CTGGTTTGGAAAGTGAAG (right PGK primer), FAM-TACTTCGGCTGACTCGGCTT-BHQ1

(PGK probe),CATCGACCAGAAGAAGTC (left SQPV primer), GCTGATGCACTTGATGAA (right SQPV primer), (TexR-CGTGTTCAACTTCCACCTCTACG-BHQ2 (SQPV probe) (primers and probes supplied by Eurofins MWG Operon, Edersberg, Germany). For more detailed information on the assay see Dale *et al.* (2016). Each DNA extract/sample was run in triplicate with a single no-template-control for each sample. A positive result was recorded when a sample showed amplification in >2/3 reactions. A sample was considered negative if no amplification occurred in 3/3 reactions. If a sample showed amplification in 1/3 reactions the analysis was repeated and the previous scoring methodology applied. If an identical result occurred the individual was categorised as inconclusive.

Epidemiological Analysis

Demonstrating the absence of an infection from a population is problematic since it would require the testing of all individuals with a test that had both 100% sensitivity and specificity. To overcome this, we estimated the confidence of freedom from SQPV infection based on the number of individuals in our sample that tested negative. More specifically, we estimated the herd-level negative predictive value (Eq. 1), which corresponds to the probability that an infection is truly absent given that a determinate number of individuals from that herd (i.e. population) are all negative to a specific diagnostic test (Martin, Shoukri & Thorburn 1992; Christensen & Gardner, 2000; Humphry, Cameron & Gunn 2004).

Eq. 1 $$HNPV = \frac{(1-eP)\times HSp}{(1-eP)\times HSp + eP\times(1-HSe)}$$

where eP is the expected prevalence and HSp and HSe are herd-level specificity and sensitivity, respectively

Estimation of the herd-level negative predictive value does not require much information about the target population, as it depends only on the expected prevalence and herd-level specificity and sensitivity, which in turn will depend exclusively on sample size and sensitivity and specificity of the chosen diagnostic test (Eq. 2 and 3). Consequently, this method can be applied to estimate confidence of freedom from a disease *a posteriori*, when sampling is restricted by field limitations or a precise estimation of population size is not available, as is often the case with wild populations.

Eq. 2 $$HSe = 1 - \left[\left(1 - (eP \times Se) + (1 - eP) \times (1 - Sp)\right)\right]^N$$

Eq. 3 $$HSp = Sp^N$$

where N is sample size and Se and Sp are the sensitivity and specificity of the test, respectively.

Based on the prevalence range observed in grey squirrels in Great Britain, where SQPV is endemic, we assumed two different scenarios and calculated the population-level negative predictive value for an expected prevalence of either 25% or 50%. In absence of specific estimates of tests performances, we conservatively assumed a sensitivity and specificity of 99% for PCR, and a sensitivity of 90% for ELISA. Specificity of ELISA was set at 92%, based on results obtained by Sainsbury et al. (2000), who found that 7.5% of SQPV positive sera reacted also to vaccinia virus.

Results

Serological Analyses

The results of the analysis of 285 sera samples are presented in the form of a histogram (Fig. 2), demonstrating the range of corrected OD_{450} values. Only 4 samples (1.4%; 95% CI: 0.04% – 2.8%) from the Italian grey squirrels gave readings > 0.2 (range 0.212 to 0.494) and therefore were considered as potentially positive for exposure to SQPV.

Molecular Analyses

Sixty-six swabs were analysed. Two swabs failed to amplify any grey squirrel PGK so they were excluded from the analysis. Four swabs showed amplification of the SQPV target gene one out of three reactions, but repeating the PCR analysis on these DNA extracts showed no amplification and skin samples from these individuals eventually proved negative, with one individual only showing one reaction out of three on both lip and flank skin. Again, subsequent analysis showed no amplification of the SQPV target gene, meaning that all the examined samples can be considered as negative.

Epidemiological Analysis

Based on 281 individuals testing negatively to ELISA, and considering the 4 positive reactors as false positive for reactivity to SQPV, the confidence of freedom from SQPV of Italian grey squirrel populations is 100% in the 50% and 25% prevalence scenario. Molecular analysis provided consistent results: considering the 64 individuals testing negative by PCR, the confidence of freedom from SQPV is either 100% assuming a 50% prevalence, or periodic 99.9% assuming a 25% prevalence.

Discussion

Demonstrating the absence of SQPV

Serological testing of Italian grey squirrels resulted in four animals out of 285 identified as potentially positive for SQPV antibodies, however in the second survey through molecular methods we did not directly detect any SQPV DNA. Based on a set of reasons that are detailed in the following paragraphs, we are confident that the four seropositive individuals may have been the

result of an ELISA cross-reaction and that our sampled squirrels were not infected by SQPV. Indeed, based on our sample size and assuming the four reactors as false positives, confidence of freedom from SQPV in the sampled populations in northern Italy is higher than 99.9%.

The squirrelpox ELISA that we applied on our samples was developed in the UK (Sainsbury *et al.*, 2000) for determining the proportion of the grey and red squirrel populations that had been exposed to SQPV. As a consequence of being uncertain about whether or not SQPV was present within Italy, even if no clinical cases had been found or had been suspected, it was not possible to revalidate the test with known negative Italian squirrel sera samples and therefore we used the OD_{450nm} 0.2 cut-off that had been established in the UK (Sainsbury *et al.*, 2000). Based on the frequency distribution of the resulting ODs, the 0.2 cut-off seems to work reasonably well for the Italian serum samples, since 98.6% of samples gave readings < 0.13 and only 4 samples (1.4%) gave readings > 0.2, with no OD readings between 0.13 and 0.2. As a result, we identified only these 4 samples as potentially coming from squirrels exposed to SQPV. It is known however that up to 7.5% of the grey squirrel sera which tested positive in the squirrelpox ELISA developed by Sainsbury and colleagues also cross-reacted in ELISA tests designed to detect vaccinia virus, suggesting sera cross-reactivity to a related poxvirus protein (Sainsbury *et al.*, 2000). It is also known that rodents, including several squirrel species, can be infected by a variety of orthopoxviruses (Emerson *et al.*, 2009; Obon *et al.*, 2011; Himsworth *et al.,* 2013; Martínez-Duque *et al.*, 2014; Wibbelt *et al.*, 2017) and therefore it is a possibility that the 4 squirrel sera exhibiting OD_{450} values >0.2 reported in this study are a reflection of these squirrels having been exposed to a different poxvirus. Indeed, a red squirrel from Spain (Obon *et al.*, 2011) and several from Germany (Wibbelt *et al.*, 2017) are known to have succumbed to infections from poxviruses that are distinct from SQPV, but whether these viruses are present in Italy, can also infect grey squirrels and would be cross-reactive in the ELISA is unknown. Due to their low specificity, indirect methods based on antibody detection are considered insufficient to officially confirm the presence of an infection in an area and the detection of the etiological agent is normally required (Guberti, Stancampiano & Ferrari, 2014). In our case, tissues

taken from squirrels covering the range of serological results, specifically including the area where two out of four suspect positive ELISA samples had originated from, were analysed for the presence of SQPV DNA, but all were found to be negative. The failure to find SQPV DNA does not mean however that these particular squirrels had not at some stage in the past been infected with the virus, as it would be expected that antibodies against the virus would be detectable for a much longer time period than the viral DNA given that most poxvirus infections tend to be acute. However, we also never found any lesions compatible with SQPV infection in over 2900 grey and 500 red squirrels examined in the field since 2011 (Wauters *et al.*, personal communication). Further support for the four query positive grey squirrels not having been exposed to SQPV comes from the fact that they are from three different locations within Piedmont and represent just 2/49 of samples from BC, 1/10 samples from IPL and 1/17 samples from VST (see Fig. 1). Moreover, after we found the first two positive samples in BC in 2011, we carried-out a second sampling in this same site one year later, but the new individuals all tested negative. Studies within Great Britain have shown that in areas where seropositive grey squirrels have been established for decades, the prevalence of SQPV can approach 100%, whereas in areas where seropositive grey squirrels had only relatively recently emerged the SQPV prevalence was lower (Sainsbury *et al.*, 2000). In Ireland, there was a progressive increase in overall seroprevalence in the woodlands analysed: from 17% in 1997/99, to 34% by 2004/2005 and 67% in 2009, a few years before the first diseased red squirrels were observed (McInnes *et al.*, 2013). Crouch *et al.* (1995), proposed that where a species was acting as a reservoir of poxvirus infection it would be expected to find >8-12% seroprevalence of antibodies to the virus. All the four query positive samples in our study had been collected within the Piedmontese population, which has been expanding for over 50 years. Therefore, if the founder animals of these longer established populations had been infected with virus it is reasonable to assume that they would exhibit a high seroprevalence if the virus was still circulating within the population.

Determining freedom from a disease in wild animal populations is among the main challenges that wildlife diseases operators and researchers have to cope with. Demonstrating the absence of a disease is generally more difficult that proving its presence, but in wildlife populations it often means dealing with a significant lack of information on both the pathogen (e.g. its origin and expected prevalence) and the host (e.g. population size). These limits imply that the application of traditional surveillance approaches originally developed on domestic animals is often ineffective (Guberti *et al.,* 2014), encouraging the development of alternative methods in order for wildlife diseases to be properly monitored and managed. In our case, following diagnostic testing, we calculated the population-level negative predictive value of each test in order to estimate, based on results obtained, the confidence of freedom from SQPV. This simple analytical method allows us to support with a 99.9% probability the absence of the infection from the sampled north Italian populations.

No SQPV in Italian squirrels: causes and implications

The fact that SQPV appears not to have been introduced to Italy is not surprising since introduction of parasites by alien hosts is largely a stochastic process and many species are commonly lost during invasion (Torchin *et al.*, 2003; MacLeod *et al.*, 2010). For example, grey squirrels in Italy and Great Britain harbour fewer macroparasite species than in their native range and the composition of their parasite communities in the two areas is also quite different (Romeo *et al.*, 2014; Romeo, Wauters & Ferrari, 2016). SQPV could also have reached Italy with founders, but could have burned out during the initial stages of the invasion due to low host densities or the absence of competent vectors. We know that Italy likely had fewer introduction events compared to Great Britain (i.e. less chance of "drawing" an infected founder) and also that population growth rate and spread after the 1948 introduction have been slower than in Great Britain (Lurz *et al.*, 2001; Bertolino *et al.*, 2008, 2014). Models by Cowan *et al.* (2016) also suggest that fleas might play a fundamental role in SQPV infection persistence, perhaps acting as mechanical vectors and

facilitating its spread. In this scenario, it should be noted that the North American grey squirrel flea *Orchopeas howardi* that commonly infects both grey and red squirrels in Great Britain is instead absent from Italy (Romeo *et al.*, 2014). Additionally, although grey squirrels in Italy acquired the red squirrel flea *Ceratophyllus sciurorum*, flea prevalence observed in Italian populations is lower than that reported in Great Britain (Romeo *et al.*, 2014, 2016).

Demonstrating that Italian grey squirrel populations are free from SQPV has important biological and management implications. Firstly, SQPV absence from Italy further suggests that the virus was introduced to Great Britain and Ireland by grey squirrels and does not have a European origin (Shuttleworth *et al.*, 2014). It has been hypothesised that SQPV initially spilled over from other native rodents, with grey squirrels then amplifying its circulation, but if this were the case, it is reasonable to assume that the infection would have emerged also in Italy.

Moreover, our findings support the notion that SQPV is a critical driver in red-grey squirrel competition, as the higher rate of grey squirrel spread (and red squirrel replacement) observed in Great Britain might be ascribed to competition mediated by disease. As predicted by models (Tompkins et al., 2003), the absence of such infections from Italian populations also confirms that mere competition for resources, albeit being a slower process, might result in red squirrel extinction by itself (Wauters *et al.*, 2005; Bertolino *et al.*, 2014; Gurnell *et al.*, 2015). These differences between introduction ranges, highlight how knowledge about disease status is essential to correctly predict spread of invaders and implement proper management and conservation strategies (Strauss *et al.*, 2012; Tompkins *et al.*, 2015).

Perspectives on red squirrel conservation

Since SQPV speeds up red squirrel replacement, its absence from Piedmont and Lombardy regions gives more time to the ongoing control activities, supporting the rate of grey squirrel spread predicted by models (Tattoni *et al.*, 2006; Bertolino *et al.*, 2008). Furthermore, considering that in

mainland Europe grey squirrel populations are present only in Italy, our findings are good news for the conservation of the red squirrel at the whole continental scale.

However, since several aspects regarding SQPV origin and its dynamics of transmission and maintenance are still undefined, a future emergence of the virus in Italy cannot be completely ruled out. In particular, we cannot completely exclude that SQPV exists in isolated areas that were not sampled in the present survey, and it should also be noted that grey squirrels were sold in Italy as pets until 2013, when a decree banned their trade, therefore a future illegal release of animals from the pet trade cannot be excluded either. Consequently, a disease surveillance plan aimed at an early detection of the virus should be set up. An active surveillance plan that requires the recurrent testing of biological samples through both serology and PCR does not appear feasible due to the large number of individuals and the high costs required to guarantee its efficacy in early detection (Guberti *et al.*, 2014). For infections with evident clinical signs such as SQPV, a passive surveillance plan that only includes the diagnostic testing of suspected cases would have a higher probability of detecting a new emergence (Guberti *et al.*, 2014). In the case of SQPV, we propose as suspect case any sciurid displaying cutaneous lesions. Efforts should thus be directed at developing an effective network for the recovery in the field of such individuals and their subsequent diagnostic testing (Everest *et al.,* 2017). Finally, additional attention aimed at an early detection of symptomatic sciurids should be given to those Italian areas where new grey squirrel nuclei may potentially establish (Mori *et al.*, 2016), even if most of them likely derive from translocations from the same Piedmontese population that was extensively sampled during the present survey (Signorile *et al.*, 2014a, 2016).

Concluding remarks

The present field survey supports with high probability the absence of SQPV infection in Italian squirrel populations and has important implications for the management of alien grey squirrels and the conservation of red squirrels in their native range. At the same time, our results confirm the role

played by SQPV in the dramatic decline of red squirrel populations in Great Britain, opposed to the slower rate of replacement observed in Italy, thus highlighting that diseases might play a critical role in biological invasions. However, given that we cannot completely be certain of SQPV absence from Italy, we recommend the future activation of health surveillance plans in order to keep the whole continental red squirrel population safe from further threats represented by infectious diseases.

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Figure 1. Location of sites in Piedmont and Lombardy regions, northern Italy, where grey squirrels (*Sciurus carolinensis*) were sampled between 2011 and 2014 to investigate squirrelpox virus infection. Line patterns indicate grey squirrel range in 2015.

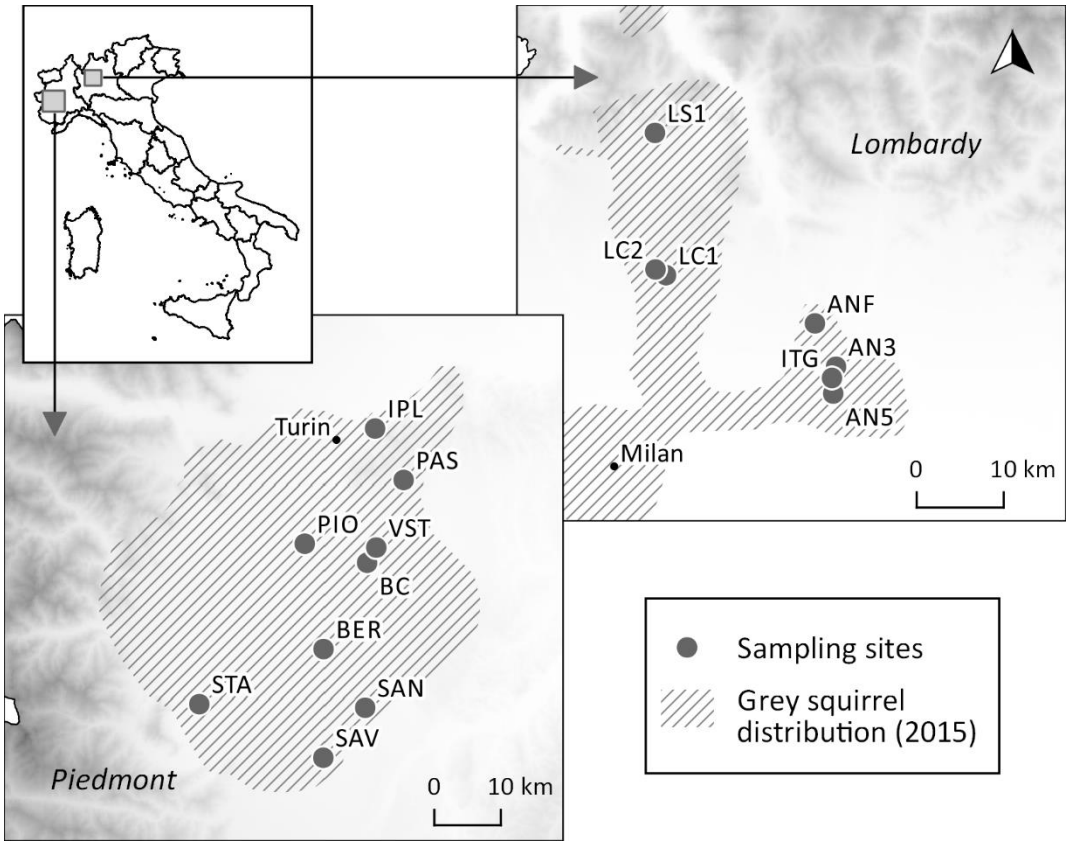

Figure 2. Frequency distribution of ELISA optical densities obtained on grey squirrel sera (N=285) tested for antibodies against squirrelpox virus. The dashed line indicates the cut-off value.