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Parapoxvirus causes a deleterious disease in red squirrels associated with UK population declines

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The disease implications of novel pathogens need to be considered when investigating the ecological impact of species translocations on native fauna. Traditional explanations based on competition or predation may often not be the whole story. Evidence suggests that an emerging infectious disease, caused by a parapoxvirus, may be a significant component of the impact that the introduced grey squirrel has had on UK red squirrel populations. Here we validate the potential role of parapoxvirus by proving that the virus is highly pathogenic in the red squirrel while having no detectable effect on grey squirrel health.

Keywords: ecological replacement; emerging infectious disease; grey squirrel; *Sciurus carolinensis*; *Sciurus vulgaris*; virus

1. INTRODUCTION

The spread of the grey squirrel (*Sciurus carolinensis*) over much of the UK during the 20th century, and the concurrent decline in red squirrel (*Sciurus vulgaris*) populations, is one of the best known and best documented cases of ecological replacement of native fauna by an introduced species (Middleton 1930; Shorten 1954; Lloyd 1983). However, the process by which this replacement occurred, and is still ongoing, is not yet fully understood (Reynolds 1985; Gurnell & Pepper 1993; Kenward & Holm 1993; Rushton *et al.* 1997; Kenward *et al.* 1998; Wauters & Gurnell 1999; Wauters *et al.* 2000). One recent hypothesis is that a parapoxvirus, possibly introduced into the UK with the grey squirrel, is a significant component of the replacement mechanism (Sainsbury *et al.* 2000). This virus is believed to have little effect on grey squirrel health, but causes mortality when transmitted to red squirrels. Model simulations demonstrate that observed population changes may have been caused by such an infection (Rushton *et al.* 2000). These findings, however, are currently unfounded as there are only observations of dead individuals found with viral lesions from which to estimate pathogenicity (Scott *et al.* 1981; Sainsbury & Gurnell 1995; Duff *et al.* 1996; Sainsbury & Ward 1996).

Emerging infectious diseases (EIDs) are increasingly cited as the causative agents behind either the exclusion or extinction of wildlife populations, often providing alternative hypotheses to explanations traditionally based on competition or predation (Berger *et al.* 1998; Harvell *et al.* 1999; Daszak *et al.* 2000; Kiesecker *et al.* 2001). Due to the complex nature of such interactions, however, evidence in support of these claims is often inconclusive and in need of experimental verification (Hudson & Greenman 1998; Tompkins *et al.* 2001). A common wildlife EID scenario is where the translocation and establishment of animals into a new geographical region, often through human involvement, introduces novel pathogens to the native fauna (Harvell *et al.* 1999; Daszak *et al.* 2000). When the introduced host acts as a reservoir population from which infection can 'spill-over' to sympatric wildlife, pathogens, which would otherwise fail to persist, may instead cause the extinction of susceptible host populations (Tompkins *et al.* 2000). Such a scenario is believed to describe the action of parapoxvirus in squirrels (Rushton *et al.* 2000; Sainsbury *et al.* 2000).

Direct competition with the grey squirrel (principally over food resources) has long been considered responsible for the decline in UK red squirrel populations (MacKinnon 1978; Okubo *et al.* 1989; Kenward & Holm 1993; Kenward *et al.* 1998). Detailed studies demonstrate, however, that such interactions alone cannot account for the rate and pattern of decline observed (Reynolds 1985; Rushton *et al.* 1997). Three lines of evidence suggest that parapoxvirus may also be involved. First, the red squirrel decline has been associated with epidemic outbreaks of infectious disease (Edwards 1962; Sainsbury & Ward 1996), a disease unrecorded before the grey squirrel introduction (Sainsbury & Gurnell 1995), and for which similarities in clinical signs suggest parapoxvirus as a common causative agent (Vizoso 1968; Scott *et al.* 1981; Duff *et al.* 1996). Second, grey squirrel seroprevalence to parapoxvirus is high in English and Welsh populations, where the red squirrel is almost extinct, but zero in Scottish and Irish populations, where the decline is far less marked and epidemic outbreaks of infectious disease have not been documented (Bryce 1997; O'Teangana *et al.* 2000; Sainsbury *et al.* 2000). Third, although infection by parapoxvirus has been frequently demonstrated for dead red squirrels (Scott *et al.* 1981; Sainsbury & Gurnell 1995; Sainsbury & Ward 1996), only one grey squirrel has been found with clinical symptoms of parapoxvirus infection (Duff *et al.* 1996).

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This implies that parapoxvirus spill-over from grey squirrel reservoirs of infection, in which the virus has little impact, may deleteriously affect sympatric red squirrel populations. Here we provide, to our knowledge, the first experimental test of this claim, investigating whether parapoxvirus is indeed highly pathogenic in the red squirrel while having little effect on grey squirrel health.

2. METHODS

Squirrel parapoxvirus disease manifests as skin lesions—erythematous exudative dermatitis and ulceration with some lesions covered by haemorrhagic scabs (Scott *et al.* 1981; Sainsbury & Gurnell 1995; Duff *et al.* 1996; Sainsbury & Ward 1996). Skin lesions were taken from six red squirrels found dead in the wild during disease outbreaks in Thetford Chase (England) between 1993 and 1996. These lesions were ground down in phosphate buffered saline to a virus titre (as determined by scanning electron microscopy (SEM)) of *ca.* 100 000 parapoxvirus particles per millilitre, and used to challenge naive individuals of both species. Naivety was confirmed by collecting no more than 1 ml of blood from the femoral vein of each individual prior to the challenge and screening sera for antibodies to parapoxvirus by direct enzyme-linked immunosorbent assay (ELISA) (Sainsbury *et al.* 2000).

In the first experiment, six grey squirrels (three male and three female) were challenged by the application of 0.2 ml lesion homogenate virus to scarification sites on both the left shoulder and left thigh, while six control grey squirrels (three male and three female) received a challenge of sterile water alone. A further six individuals (again, three male and three female) were challenged with red squirrel parapoxvirus cultured in foetal lamb muscle cells, to assess the degree to which this stock of virus had attenuated. As the serological responses observed in this experiment were not as high as expected (see § 3), a larger challenge to the same individuals was conducted eight months later to ensure that our test of parapoxvirus impact was valid (in that all experimental individuals were successfully challenged). Each individual received the same inoculum as before, with 0.2 ml being applied to a scarification site on the right thigh, 0.2 ml injected subcutaneously into the right shoulder and 0.1 ml administered to each nostril.

In the second experiment, four naive captive-bred red squirrels (two male and two female) were experimentally challenged with lesion homogenate virus while a further four (again, two male and two female) were sham challenged with phosphate-buffered saline containing no virus. Captive-bred individuals were used as wild red squirrels are protected in the UK. Before the experiment, a sample size of four animals per group was decided upon as the minimum number with which the expected effects of parapoxvirus on red squirrels could be meaningfully demonstrated. Individuals were challenged by the application of 0.2 ml homogenate to a scarification site on the right thigh and a subcutaneous injection of 0.2 ml homogenate into the right thigh. As deleterious effects were possible, a clinical scoring system was drawn up, based on all available background knowledge and advice with which to score the extent of the disease impact on each individual (both experimentals and controls) every 12 h over the course of the experiment (table 1). A clinical score of eight points was agreed on before the trial as the level at which disease effects would almost certainly cause mortality in the wild. To provide a humane end-point, it was decided *a priori* to remove from the experiment, any individual with a cumulative score of eight or more on three consecutive occasions.

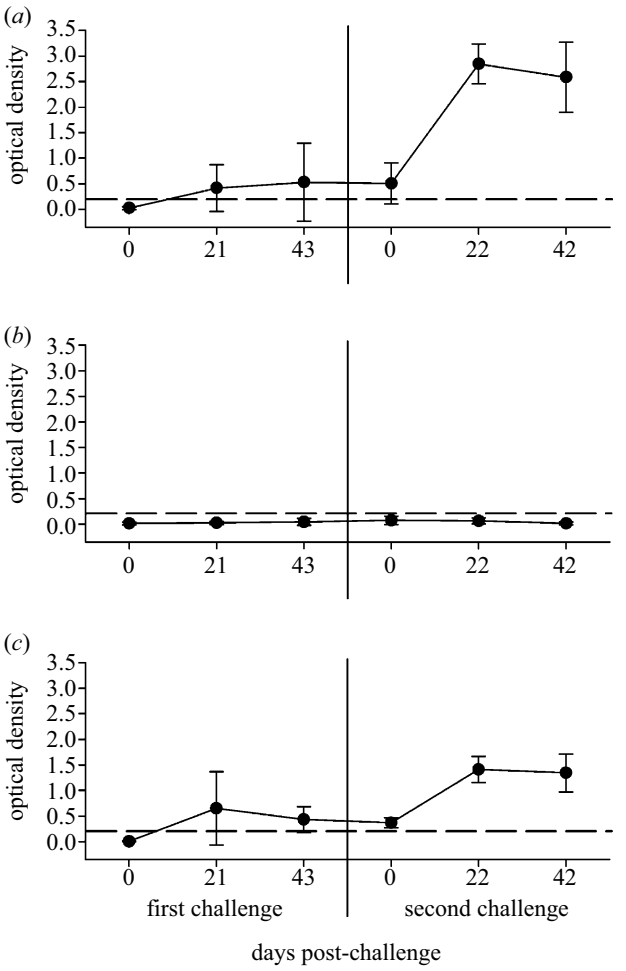

Figure 1. Corrected optical density values for a direct ELISA testing for the presence of antibodies to parapoxvirus in sera taken from (*a*) six grey squirrels challenged with lesion-homogenate virus, (*b*) six control grey squirrels sham-challenged with sterile water and (*c*) six grey squirrels challenged with cell-cultured virus. All individuals were challenged twice, with the second challenge occurring eight months after the first challenge. Values shown are means ± 1 s.d. The horizontal dashed line indicates the cut-off point for a positive result (OD value of 0.20).

To monitor antibody responses, femoral blood samples were collected from all individuals at three and six weeks following each challenge in the first experiment, and at two, four, six and eight weeks post-challenge during the second experiment. Blood was also taken when any individual was removed from an experiment. One sham-challenged red squirrel was removed from the second experiment at four weeks post-challenge to provide an uninfected control with which to compare the pathology and histopathology of any virus-challenged individuals removed prior to that point.

3. RESULTS

Previous work indicates that the cut-off point for a positive result in the direct ELISA for parapoxvirus antibody (protein G conjugate) is an optical density (OD) of 0.20 at 492 nm, with OD values reaching as high as 2.20

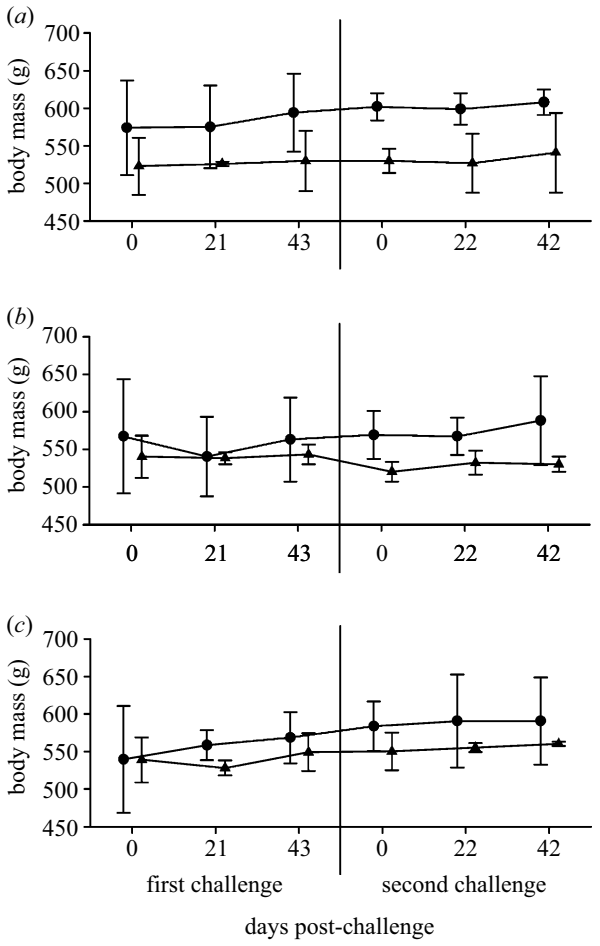

Figure 2. Comparison of body mass between (*a*) grey squirrels (three male and three female) challenged with lesion-homogenate parapoxvirus, (*b*) control grey squirrels (three male and three female) sham challenged with sterile water and (*c*) grey squirrels (three male and three female) challenged with cell-cultured parapoxvirus. All individuals were challenged twice, with the second challenge occurring eight months after the first. Values shown are means ± 1 s.d. Circles denote male squirrels and triangles denote female squirrels.

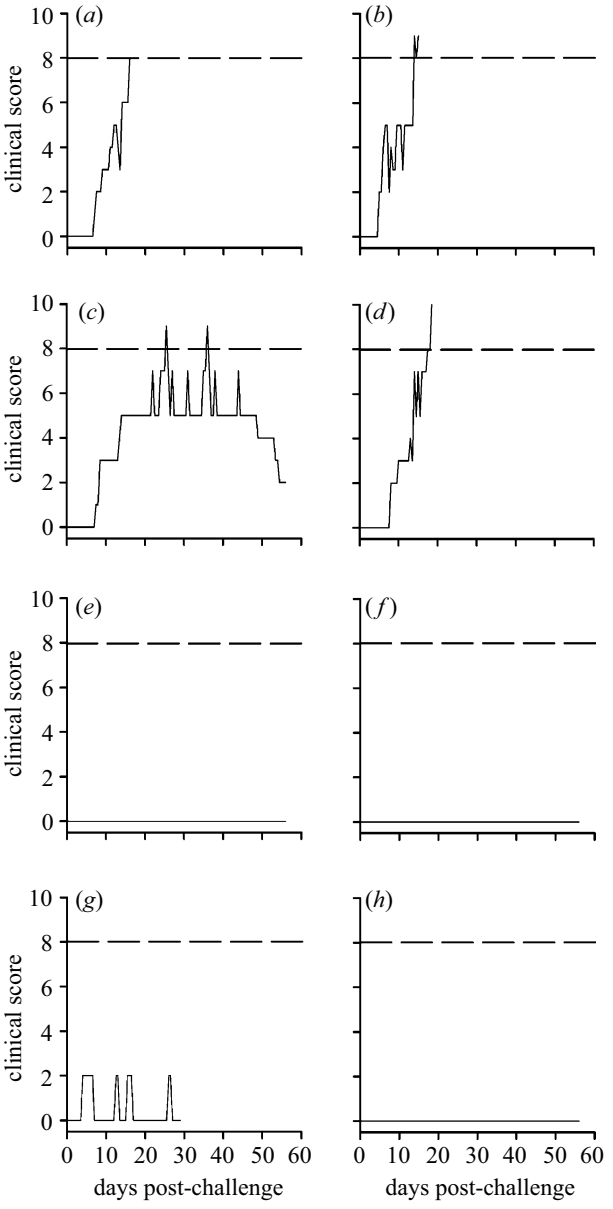

Figure 3. Clinical scores (see table 1) for (*a–d*) four red squirrels challenged with lesion-homogenate parapoxvirus, and (*e–h*) four control red squirrels sham-challenged with phosphate-buffered saline. The horizontal dashed line indicates the score at which disease effects would almost certainly cause mortality in the wild (score of eight). Individuals which equalled or exceeded this level on three consecutive occasions (at 12 h intervals) were removed from the experiment. One sham-challenged individual (*g*) was removed from the experiment after 28 days as a control for the pathological and histopathological examination of the virus-challenged individuals.

(Sainsbury *et al.* 2000). The serological response of the grey squirrels challenged with parapoxvirus was thus not as high as expected (figure 1), although 11 out of the 12 individuals did exhibit an antibody response of OD 0.20 or greater at least once when sera were screened at 21 and 43 days post-challenge.

The serological response to the second challenge was far greater (figure 1). While OD values for the control grey squirrels remained well below 0.20 over the course of both challenges, squirrels challenged with the virus for a second time exhibited an antibody response of at least OD 1.07. Whether the stronger response was due to either the challenge technique being more effective or the priming of the immune response by the first challenge is not known. It does demonstrate, however, that each experimental grey squirrel was successfully challenged at some time over the course of the study. The serological response of individuals challenged with lesion homogenate virus was significantly greater than that of those challenged with cell-cultured virus at both 22 and 42 days after the second challenge (repeated-measures ANOVA $F_{1,10} = 29.68$, $p < 0.001$). Thus, attenuation of the cell-cultured virus may have occurred.

All 18 grey squirrels were in good condition both prior to and during the two experimental challenges, with no clinical signs of parapoxvirus infection being observed at any time. Furthermore, there were no significant differences in body mass (controlling for sex) among the experimental and control groups, either prior to (ANOVA $F_{2,12} = 0.11$, $p = 0.89$), or during (repeated-measures ANOVA $F_{2,12} = 0.34$, $p = 0.72$) the challenges (figure 2).

Table 1. Clinical scoring system used to assess the impact of parapoxvirus on experimentally challenged red squirrels. (Scores from each category were combined to provide a cumulative total reflecting the magnitude of disease effects.)

assessment category	impact observed	clinical score
size of parapoxvirus lesions	$<$ 1 mm diameter	1
	$\geq$ 1 mm and $<$ 3 mm diameter	2
	$\geq$ 3 mm diameter	3
parapoxvirus lesions at locations other than the challenge site	one other location	1
	two or more other locations	2
depression	animal 'sick' looking (lethargic, poor condition)	1
	appetite loss ($>$ 50% reduction in food consumed)	2
	complete loss of appetite	3
weight reduction after experimental challenge	$\geq$ 10% and $<$ 20% weight loss	2
	$\geq$ 20% and $<$ 30% weight loss	4
	$\geq$ 30% weight loss	8

This lack of virus impact is in direct contrast with the effects observed when red squirrels were challenged. While none of the sham-challenged red squirrels scored more than 2 on the clinical scoring system (always due to periods of 50% appetite loss, probably caused by the stress of handling and captivity), all four virus-challenged red squirrels scored in excess of 8 points within the first four weeks of the experiment (figure 3). This was due to the development of severe lesions (the same or greater than 3 mm in diameter), the appearance of secondary lesions (lesions at locations other than the challenge site) within two weeks of the challenge, lethargy and poor condition, loss of appetite of up to 100% and weight loss of up to 30%. All four challenged individuals exceeded a clinical score of 8 points, whilst no control individuals scored more than 2 (figure 3), making the demonstrated impact of parapoxvirus statistically significant (Fisher's exact test $p = 0.014$). Detailed pathological and histopathological examination of the four virus-challenged red squirrels revealed no internal signs of disease.

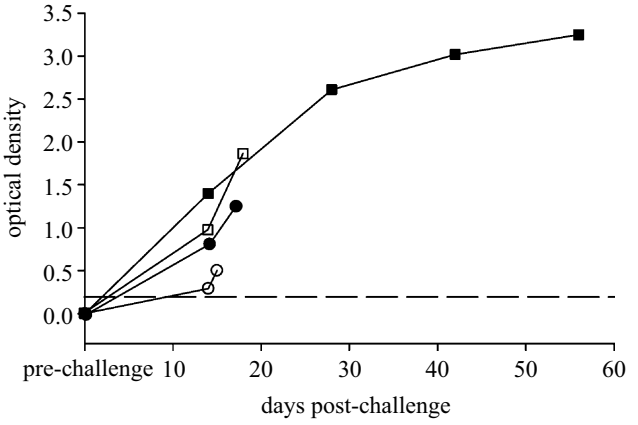

Figure 4. Corrected OD values for a direct ELISA testing for the presence of antibodies to parapoxvirus in sera, taken from four red squirrels challenged with lesion-homogenate virus. (With reference to figure 3, closed circles denote individual (*a*), open circles (*b*), closed squares (*c*), and open squares (*d*)). The horizontal dashed line indicates the cut-off point for a positive result (and OD of 0.20). Sera was also taken from four control red squirrels, whose antibody responses never exceeded an OD value of 0.04 over the course of the experiment.

4. DISCUSSION

This study proves that parapoxvirus has the ability to cause a debilitating disease of red squirrels, whilst having no apparent impact on challenged grey squirrels. The clinical signs documented here (including lesions around the face, ventral skin surfaces of the feet and body, medial skin of the legs and the genital region) are the same as those observed on squirrels recovered dead from the wild (Scott *et al.* 1981; Sainsbury & Gurnell 1995; Duff *et al.* 1996; Sainsbury & Ward 1996), indicating that parapoxvirus was the most probable cause of mortality in the latter instances.

Three out of the four virus-challenged red squirrels were removed from the experiment before it reached its conclusion (set, *a priori*, at eight weeks post-challenge) as they equalled the end-point condition of a clinical score of 8 points or more at three consecutive occasions (figure 3). This level of deterioration occurred within 1–4 days of secondary lesions appearing, suggesting that the potential for the transmission of infection from these individuals to other individuals in the wild would be limited. The fourth virus challenged individual, however, did not score 8 or more points until three and a half weeks post-challenge and never equalled or exceeded a score of 8 points on more than one occasion at a time (figure 3*c*). This individual had exuding, inflammatory secondary lesions for up to six weeks post-challenge, after which it recovered. This illustrates two points. First, there may be the potential for disease transmission between red squirrels in the wild, making parapoxvirus a disease of red squirrel populations, rather than just individuals. This supports the hypothesis that spill-over of virus from infected grey squirrels, triggering localized red squirrel epidemics which eventually 'fade-out' due to the high level of mortality caused, is the mechanism behind the sporadic pattern of disease outbreaks seen in the wild (Rushton *et al.* 2000). Such a mechanism would facilitate the movement of grey squirrels into habitat previously occupied by red squirrels, and may account for the patterns of ecological replacement that have occurred which direct competition hypotheses are unable to explain (Reynolds 1985; Rushton *et al.* 1997).

The second point illustrated is that red squirrels can

potentially survive infection by parapoxvirus, although the prolonged disease course observed here in one animal did occur under conditions of optimal temperature, food *ad libitum*, and the absence of predation or competition pressure, or biting ectoparasites that may irritate the skin and foster the spread of secondary lesions. A possible reason why one virus-challenged red squirrel was less affected by parapoxvirus than the other three is that its initial antibody response to the challenge (measured at 14 days post-challenge) was the highest of the four individuals by 38% (figure 4). Furthermore, its antibody response eventually plateaued at a level similar to both that observed in grey squirrels in the wild (Sainsbury *et al.* 2000) and that seen in the six homogenate-challenged grey squirrels (figure 1), individuals in which the virus had no clinical effects (figure 2). A related observation is that the virus-challenged red squirrel with the lowest initial antibody response (lowest by 48%; figure 4) was the individual in which disease development was fastest, being the first to reach a clinical score of eight points (figure 3*b*). These observations suggest that the development and application of a red squirrel vaccine against parapoxvirus, as has been discussed in the literature (Rushton *et al.* 2000; Sainsbury *et al.* 2000), has a good chance of conferring a high degree of immunity to the disease caused by the virus and may thus be an essential management tool in the effort to conserve the remaining UK red squirrel populations.

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