THE UNIVERSITY
of EDINBURGH

A study of squirrelpox virus in red and grey squirrels and an investigation of possible routes of transmission

Caterina Fiegna

Thesis submitted for the degree of Doctor of Philosophy to
The College of Medicine and Veterinary Medicine
University of Edinburgh

Research was carried out at Moredun Research Institute

2011

Author Declaration

The work and results presented therein have been conducted by the author and under the supervision of Dr. Colin McInnes, Dr. Kim Willoughby (Moredun Research Institute) and Professor Elspeth Milne (Edinburgh University). The work has not been previously submitted for any other degree or professional qualification. When relevant, acknowledgement has been made of collaboration with other colleagues.

.....

Caterina Fiegna

Contents

Acknowledgments

A most sincere thank you to my supervisors Dr. Colin McInnes and Professor Elspeth Milne for their support, patience and guidance - the study simply would not have been possible without them. Also a special thanks to Dr. Kim Willoughby for all her input.

I am very grateful to Dr. Lesley Coulter and Ann Wood, for their help in the lab, for always being available, and for the cakes during coffee breaks! Thanks also to Janice Gilray for providing the squirrels ELISA results and talking me through the ELISA procedure. A warm thanks to Mark Dagleish, who spent long hours in HSU with me to perform the post mortem on grey squirrels, for all the lifts at work, and for introducing me to the climbing wall. To David Dean for providing red squirrel antibodies and a particular thanks to Jeanie Finlayson for her immense work on immunohistochemistry. I am also obliged to Clare Underwood and Val Forbes who helped process histopathology samples. For the post mortem examination data and red squirrels material I am very grateful to Anna Meredith and the pathology staff of the Royal (Dick) Veterinary Studies in Edinburgh. Thanks to all the field workers, conservation organizations and ranger services for providing red squirrel carcasses and in particular to Brian Gibson for providing grey squirrels, and also to Louise Gibbard and all the staff from HSU for their support dealing with all the grey squirrels.

A very special thanks to Dr. Peter Lurz for always encouraging me, for the immense collection of relevant literature, his insightful comment and overview, and for never asking when I would submit!

Finally, I have also had great support from my family and friends. Thank you to Antonello – the ‘little legend’, all the members of my enlarged family and all my old friends and the new ones I have made in these last few years, for their friendship and encouragement along the way. Last, but not least, to Niall for keeping me sane in the final part of this journey.

List of Abbreviations

α	alpha
β	beta
γ	gamma
Δ	delta
%	percentage
§	section
♀	female gender
♂	male gender
µ	micro
°C	Celsius grade
ADCC	antibody dependent cell-mediated cytotoxicity
AR	antigen retrival
bp	base pair
BPSV	*Bovine papular stomatite virus*
C	cytosine
ca.	*circa*
CEV	cell-associate enveloped virus
ChPV	*Chordopoxvirinae*
CMI	cell-mediated immunity
Cp	crossing point
CPE	cytopathic effect
CPXV	*Cowpox virus*
CTL	cytolytic T cell
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTPs	deoxyribonucleotides
DPI	day(s) post infection
dsDNA	double-stranded DNA
E%	efficiency percentage

E. coli	*Escherichia coli*
e.g.	*exempli gratiā*
ECTV	*Ectromelia virus*
EDTA	ethylenediamine tetraacetic acid
EEV	extracellular enveloped virus
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
FLM	foetal lamb muscles
FLSc	foetal lamb skin cells
G	guanine
g	gram(s)
g	gravity force
H&E	haematoxylin and eosin
H_2SO_4	sulphuric acid
HRP	horse radish peroxidase
ID	identification
IEV	intracellular enveloped virus
IFNs	interferons
IgG	immunoglobulin G
IMV	intracellular mature virion
ITR	inverted terminal repeat
IV	immature virion
JSRV	*Jaagsiekte sheep retrovirus*
kbp	kilobase pair
KCl	potassium cloride
LB	Luira Bertani broth
l	litre
LH	left hand
LSDV	*Lumpy skin disease virus*
m	meter
M	molar
MCH	major histocompatibility complex

mg	milligram
min	minute(s)
ml	milliliter
mM	millimolar
MOCV	*Molluscum Contagiosum virus*
MPXV	*Monkeypox virus*
MYXV	*Myxoma virus*
n	nano
N	number
NK	natural killer cell
NS	no significant
OD	optical density
OPV	*Orthopoxvirus*
p.i	post infection
ORFV	*Orf virus*
PCPV	*pseudocowpox virus*
PCR	polymerase chain reaction
PK	proteinase K
qPCR	Real Time PCR
r	Pearson's correlation number
R^2	coefficient of determination
RNase	ribonuclease
rpm	revolution per minute
RT	room temperature
SD	standard deviation
SF	Squirrel fibromatosis
SPPV	*Sheeppox virus*
SPV	Sealpox virus
SQFV	Squirrel fibroma virus
SQPV	Squirrelpox virus
Taq	*Thermus aquaticus*
UV	ultraviolet

v/v	volume/volume
VACV	*Vaccinia virus*
VARV	*Variola virus*
w/v	weight/volume

Abstract

The red squirrel (*Sciurus vulgaris*) is native to Eurasia, but in the UK its survival is being threatened by the non-native grey squirrel (*Sciurus carolinensis*). Since its introduction to the UK from the USA the grey squirrel has increased its range at the expense of the red squirrel. Although competition for resources clearly plays a role in this replacement, an infectious viral disease, caused by squirrelpox virus (SQPV) and hosted apparently asymptomatically by the grey squirrels, has now been recognised as a major contributing factor.

Little is known about the pathogenesis of infection in grey squirrels in comparison to red squirrels, but understanding this is essential to determining how the virus spreads within and between the red and grey squirrels. The aims of this thesis were to investigate the course of SQPV infection in red and grey squirrels and possible routes of virus transmission. Specifically, for the first time, a novel Real Time PCR (qPCR) assay and immunohistochemistry were used to investigate the presence of SQPV in various tissues from naturally infected red squirrels and experimentally infected grey squirrels.

In diseased red squirrels SQPV DNA was found in several tissues with the highest amounts being found in skin samples. This reflects the multiple lesions that were easily visible on the red squirrel carcasses. There was no indication of systemic disease although the viral DNA was detected, at lower levels, in other internal organs. Grey squirrels were experimentally infected with SQPV isolated from naturally-infected red squirrels with fatal clinical disease. In contrast to SQPV-infected red squirrels no clinical lesions, other than mild scab formation at the site of inoculation, were found in the grey squirrels post-infection. No gross pathological changes indicative of systemic infection were observed and these findings were reflected in the qPCR and histopathology results. Viral DNA was only detected by qPCR in samples from the site of inoculation (scarified skin) and at lower concentrations in other skin tissues such as digital and eyelid skin. In addition, histopathology and immunohistochemistry examination revealed evidence of infection characterized by ballooning degeneration of keratinocytes, and acanthosis

and spongiosis of the epidermis. These skin lesions were self limiting and minor compared to the infected red squirrel skin samples.

The molecular variation in the virus isolated over time from different parts of the UK was also investigated. Seven SQPV isolates (4 from Scotland and 3 from England) were tested and results indicated that there are no significant changes in the amino acid sequence of any of the three genes examined apart from one amino acid change (one base change) in one gene. All Scottish isolates examined showed this change in comparison to English isolates.

The results in this thesis show that there is a mild pathology associated with SQPV infection in grey squirrels. Scabs form at the site of infection but are less proliferative than in infected red squirrels, though they may still serve to contaminate the environment with virus leading to further outbreaks of disease. In contrast it seems likely that the proliferative lesions suffered by red squirrels and the greater amounts of virus that this leads to are likely to be more significant to the epidemiology of disease in localised outbreaks.

CHAPTER 1

INTRODUCTION

1.1 History of squirrels in the UK

Currently, there are two species of squirrel found in the United Kingdom (UK), the red squirrel (*Sciurus vulagaris leucourus* Kerr 1792) and the grey squirrel (*Sciurus carolinensis* Gmelin 1788). The red squirrel is the native squirrel species to the British Isles, whereas the grey squirrel was introduced from America in the mid to late 19th century. Within a few decades of its introduction to various points within England the grey squirrel had spread throughout the forests and woods of the country with dire consequences for the native red squirrel (Shorten, 1954; Lloyd, 1983).

1.1.1 Grey squirrels in the UK pre-1930

The natural history of the grey squirrel in the UK is complex. It is possible that grey squirrels were introduced to the UK at the beginning of the nineteenth century, but it is difficult to ascertain the accuracy of early sightings. Anecdotal reports of the presence of grey squirrels in the country came from a letter to the "Cambrian Quarterly Magazine" in 1830 (Middleton, 1930) which gives a description of a squirrel resembling the North American grey squirrel in Montgomeryshire and Denbighshire. Similarly, a letter to "Country Life" in 1929 speculated that grey squirrels may have been seen near Canterbury since the middle of the 19th Century (Middleton, 1930). The first authenticated record, however, of a documented release of imported American grey squirrels was at Henbury Park, Cheshire, where two were released by a Mr. Brocklehurst in 1876 (Middleton, 1930).

The present distribution of grey squirrels in the UK is thought to be due to the various introductions from 1890 onwards, many from the most important stock of American grey squirrels brought to this country in 1889 by Mr. G. S. Page of New Jersey, and which were used to found a large colony at Woburn Park, Bedfordshire (Middleton, 1930; Middleton, 1931). So successful were the squirrels at Woburn that they were then used to found colonies across the UK as well as Ireland (in 1913), and

South Africa. Another significant introduction appears to have been in 1902 at Kingston Hill, Surrey where large numbers of grey squirrels were released.

The first evidence of grey squirrels in Scotland dates back to 1892 when three animals imported from Canada were released at Finnart on Loch Long (Middleton, 1931). For ten years after that there is no record of any more introductions, although in the east of Scotland, some grey squirrels were introduced at some stage to the Zoological Gardens in Edinburgh from an unknown source. By 1913 some of these had escaped the confines of the zoo and had begun to spread. After few years, more squirrels were introduced to in Dunfermline, in 1919 (Shorten, 1954).

According to Shorten (1954), between the years of 1876 and 1929, a total of thirty-one grey squirrel introductions took place, although Lloyd (1983) suggests that grey squirrels were not imported to the UK from the US after 1915. Instead, local introductions were mostly translocations from established colonies and as a result the grey squirrel rapidly expanded its range throughout central and southern England, Wales and Scotland (Shorten, 1954).

There are at least five subspecies of *Sciurus carolinesis* within the group of tree squirrels native to America and Canada, each with a different distribution. The grey squirrel (*Sciurus carolinensis* Gmelin 1788) is found mainly in the eastern US and is also known as the Eastern grey squirrel. Its natural range extends west to the edge of the deciduous forest and north to Canada, but it has been translocated within the US to California, Montana, Oregon and Washington and also to several regions of Canada (Koprowski, 1994).

The present population of grey squirrels in the UK is thought to represent the merging of colonies derived from two subspecies *(Sciurus carolinensis carolinensis* and *Sciurus carolinensis leucotis*) introduced from various parts of both the US and Canada (Shorten, 1954). This would suggest that the grey squirrel population in the UK was not established from a single introduction but from repeated introductions over time from the US and Canada. Genetic analyses of grey squirrels populations in England found high levels of diversity despite the relatively recent introduction of grey squirrels in the UK and supports the notion of multiple introductions and subsequent mixing of populations (David-Gray *et al.*, 1998; Middleton, 1930).

In Europe, the Eastern grey squirrel was separately introduced to northern Italy (from Canada) (Currado, 1998; Koprowski, 1994). Grey squirrels which were descendants of those initially introduced to England were also translocated to Ireland (1913), South Africa (Lloyd, 1983; Middleton, 1930; Shorten, 1954) and Australia, but those introduced to Australia failed to establish themselves and died out within 100 years (Peacock, 2009; Shorten, 1954; Lloyd, 1983; Koprowski, 1994).

1.1.2 Red squirrels in the UK pre-1930

Sciurus vulgaris leucourus Kerr 1792 is the subspecies of Eurasian red squirrel that has been described for the UK, although it may be that current populations have also acquired the genetic influence of the red squirrel subspecies from the continent. It is known that some red squirrels belonging to the subspecies (*Sciurus vulgaris fuscoater* and *russus*) were imported from Norway and Sweden in the 17th century and that the descendants of these can probably still be found in Scotland and North-east England (Shorten, 1954; Hale & Lurz, 2003).

In Wales, the earliest reference to red squirrels was in 13th century and it seems that they were indigenous to the north of Scotland from early times until the 17th century. Shorten (1954) reports fluctuation in the number of red squirrels in Scotland during the 17th and 18th century. Citing Harvie-Brown (1878-81) she states that during that period, red squirrels went through hard times and by the end of the 18th century they had become almost extinct. One of the main reasons given for the decrease in red squirrels was increased forest destruction in order to create new sheep pastures and land for cultivation proposes. It was also suggested that severely cold winters and epidemic disease could have caused the reduction in numbers (Shorten, 1954). As a result of the severe fall in red squirrel numbers it is reported that red squirrels were re-introduced to Scotland, probably from England and Wales, although as stated above, red squirrels were also introduced from Scandinavia and possibly the Baltic States (Harvie-Brown, 1878-81).

By the end of the 19th century, possibly due to the re-introductions and increase in the number of conifer trees being grown, red squirrels again became plentiful, with numbers being reported to be at an all time high in Scotland and Wales. This actually resulted in organised red squirrel eradication campaigns. In the New Forest, control

measures were started against the red squirrel in 1880 (Shorten, 1954), because of the damage they were doing to trees. In 1889 it was reported that more than 2200 squirrels were killed in 1 year. Similarly, in the north of Scotland, the Highland Squirrel Club was formed in 1903 and over the next 30 years its members killed over 82,000 red squirrels. In 1910, another club was formed in Cornwall with the purpose of killing those that had spread from surrounding woodlands, destroying gardens in search for food.

1.1.3 Distribution of red and grey squirrels in the UK post-1930

Interest in the relative abundance and distribution of red and grey squirrels increased after it was first suggested by Middleton (1930) that the rising numbers of grey squirrels may have been having an effect on the native red squirrel population. He stated that the number of red squirrels in the country had undergone a sustained reduction since the start of the century, primarily occurring in England between 1904 and 1914, whilst in the north of Scotland, Ireland, north Wales and Cumberland the decrease was observed later in the 1920s. He conceded that this may have been part of a normal 7-8 year cycle of fluctuating population size, a phenomenon which he states was first noted by Herbert in 1832, but also suggested that the expansion of grey squirrels may also have contributed to falling red squirrel numbers. This is despite it being reported that of the 44 districts in England and Wales in which the decline in red squirrel numbers was noticed, only four were said to have had grey squirrels present at the time. This suggested that the observed decrease in the number of red squirrels occurred independently of known grey squirrel presence.

Organised surveys of the distribution of both species of squirrels have been performed over many years by Government agencies such as the Nature Conservancy Council, the Department of Agriculture in Scotland, the Forestry Commission and the Ministry of Agriculture, Fisheries and Food (M.A.F.F.), as well as by local volunteer groups. In the New Forest the decline in red squirrels became evident after 1940 and although it was attributed to the presence of the grey squirrel (greys arrived in 1940), other instances of red squirrel decline in Europe (Finland and Russia) were observed at the same time. Between 1945 and 1950 there were reports of local reductions in red squirrels in areas where the grey squirrel had been present

for at least two years, but equally reds were found dying in other areas such as Dorset after the dry summer of 1949 where no greys had been recorded (Shorten, 1954). Reviewing distribution records, Lloyd (1983) reported that in1945 it appeared that the red squirrel was more broadly distributed than the grey but by 1971 the greys occupied about four times the area compared to reds in England and Wales. This, it was suggested, may have been as a result of the number of conifer trees which were felled during the wars (1914-18 and 1939-45) thereby reducing the available refuges of the red species and because of hard winters such as that of 1946-47 which seemed to have caused a decrease in the numbers of red squirrels across the whole of the country. The observed decline in red squirrel numbers was also supported by a survey carried out by MAFF in 1971 covering areas (West Suffolk, Essex, Lincolnshire and mid south Wales, the north of England and parts of Norfolk) that had been surveyed previously in 1959. One anomaly however, which arose from the two surveys was that in 1971 red squirrels were recorded in areas where they had not been found in the previous survey. It is not known whether this increase in distribution was accurate or if it just reflected the improvements in surveying methodologies. Shorten (1954), suggests that it was likely to be the latter. It was however noted that at least since 1945, the red squirrels seemed to find it more difficult to maintain their numbers in areas where the grey squirrels were present.

Today the red squirrel population in the UK is estimated at approximately 140,000, but it is acknowledged that they are almost extinct in England and Wales, and that the majority of the population (75%) is found in the north of Scotland (Gurnell & Hare, 2008). In contrast, grey squirrels are thought to number approximately 2.5 million, spread throughout the UK, with the north of Scotland being a notable exception. The decline in red squirrel numbers has lead to concern about the conservation status of the species. The red squirrel is now officially considered endangered in the UK, being listed in the UK Biodiversity Action Plan and is also under legal protection (Schedules 5 and 6 of the Wildlife and Countryside Act 1981).

1.1.4 Evidence of the role of disease in the decline of the red squirrel in the UK

The reasons for the decline in the red squirrel population throughout the 20th century could be attributed to many variables, circumstances and reasons. For instance, it is suggested that the preferred habitat of the two species is different (red squirrels prefer conifer woodland whereas grey squirrels prefer broad leaved species of tree) and that in certain woodlands (e.g. oak dominated) one would have the competitive advantage over the other (Kenward & Holm, 1993). Indeed, competition for woodland resources was for a long time thought by some to play the major role in the replacement of the red squirrels by the grey squirrels and still plays a part (Gurnell *et al.*, 2004)

Middleton (1930), however, observed that on many occasions the reduction in the number of red squirrels in a local population was reported to be sudden, over a short period of time. As a result, he suggested that epidemic disease amongst the squirrels could be an alternative reason for the decline in numbers of red squirrels since 1900. He proposed that coccidiosis may explain the cause of death of many of the red squirrels, since it was very common in rodents and had been found in several dead squirrels from Kent between 1904 and 1926. However, the description of external lesions in dead and dying squirrels from other parts of the country suggested that it was not the only reason for red squirrel deaths. The descriptions of external disease signs often resembled those of mange, and indeed, mange had also been described in grey squirrels. In 1925 there was a die off of grey squirrels in Henbury Park, Cheshire, as a result of a disease epidemic. In this instance, the signs resembled those of mange; "grey squirrels were noticed to lose fur in patches, scrofulous skin, and tails like rats" (Middleton, 1930). Outbreaks of disease were reported in grey squirrels throughout the 1920s and it is interesting to note that Middleton (1930) was one of the first authors to suggest that the alien grey species introduced from America could be the carrier of a disease which was fatal for native red squirrels.

The debate over whether or not disease and subsequent reduction in red squirrel numbers was connected to the presence of the imported grey squirrel was one that was revisited throughout the 20th century. Edwards (1962) reported an outbreak of

fatal disease in red squirrels in Shropshire woodland in 1960 where many reds were dying of a disease of unknown aetiology with the course of illness similar to that of myxomatosis (Edwards, 1962). This was just at the time when the grey squirrels were first seen in the woodland. At that time it was not possible to connect the occurrence of grey squirrels with the disease in red squirrels; indeed it was suggested that the grey squirrels may have found it easier to move into the area and spread out rapidly specifically because the red squirrels were diseased and declining in numbers (Lloyd, 1983). Vizoso (1968) reported the occurrence of fatal disease in Norfolk from 1963-1966 and although he stated that grey squirrels had not been seen in the locality, later on he admitted that the presence of stressors and in particular the grey squirrels, may have led to the expression of overt disease in red squirrels and eventually to their local extinction (Vizoso, 1968). Keymer (1974) also investigated cases of disease in East Anglian red squirrels which eventually culminated in the identification, by electron microscopy, of a parapoxvirus from an eyelid lesion (Scott *et al.*, 1981a). Here it was reported that the squirrel in question had been seen fighting with a grey squirrel three weeks before it was found dead.

By the end of the 20th century it was established that the localised disappearance of red squirrels, in most instances, coincided with the arrival of grey squirrels in the same woodland (Gurnell, 1987), and through the work of several authors it has been generally accepted that this ecological replacement of red squirrels by the greys is most likely to be pathogen-mediated (Gurnell *et al.*, 2006; Rushton *et al.*, 2006; Sainsbury & Ward, 1996; Sainsbury *et al.*, 1997; Tompkins *et al.*, 2002; Tompkins *et al.*, 2003)

1.1.5 Pox disease in red squirrels

It is not known when poxvirus disease first became a problem for the red squirrel in the UK. In 1931, Middleton reported that a disease, the signs of which would now be attributed to poxvirus, was found in the Norfolk area in 1920. Similarly, although the aetiology was not determined, it is thought that the outbreak of disease in the Shropshire woodland in 1959/60 described by Edwards (1962) was almost certainly caused by poxvirus. Vizoso (1968) described a disease, again in Norfolk and found throughout the 1960s, that he believed was the same as that described by Edwards,

the signs being likened to those of myxomatosis. Later, Keymer (1983) described attempts to isolate the agent, presumed to be a virus, which was responsible for the myxomatosis-like disease in Norfolk (Keymer 1983). It was not until 1981, however, that a virus, described as a parapoxvirus because of its morphological similarity to *orf* virus when visualised by electron microscopy (**Figure 1.1**), was isolated from a red squirrel found dead in a Norfolk woodland in 1980 (Scott *et al.*, 1981a). As a result, poxvirus infection was suggested as a significant factor in the decline of the red squirrel population in East Anglia.

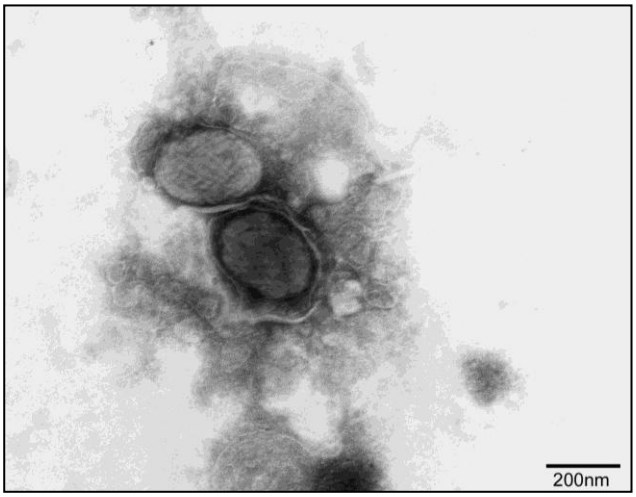


Figure 1.1 Squirrelpox virus particles.

Negative staining transmission electron microscopy of SQPV particles
Courtesy of David Everest (VLA).

Researchers subsequently came to recognise that pox disease was likely to have caused significant mortality in red squirrel populations throughout the country where the red squirrels were sympatric with grey squirrels (Sainsbury & Ward, 1996). The exact role of the grey squirrel in the manifestation of disease in the red squirrel was however questioned because it had been thought that the disease did not affect the grey squirrel. However, Duff *et al.*, (1996) reported finding a grey squirrel which exhibited signs of pox disease. Electron microscopic analysis of the virus found it to be similar to that reported by Scott *et al.*, (1981a), thus providing the first evidence

that grey squirrels were also susceptible to the parapoxvirus (now known as squirrelpox virus). It was suggested that since the grey squirrel normally appeared to be very resistant to this disease, the virus may be maintained in the grey squirrel population, from where it was transmitted to the more susceptible red squirrels (Duff *et al.*, 1996). However, the authors also conceded that it was possible that the virus was present as a subclinical or latent infection in the red squirrel and that the stress caused by the interaction with grey squirrels resulted in clinical disease.

Further evidence that supported the grey squirrels' role as a reservoir of the virus was discovered in the late 1990s. An ELISA test was developed to assess the presence of antibodies to the squirrelpox virus in the serum of both red and grey squirrels (Sainsbury *et al.*, 2000). The serological survey showed that a large proportion of the grey squirrel population in England and Wales had been exposed to the squirrelpox virus (Sainsbury *et al.*, 2000). In contrast, the majority of red squirrels did not appear to have been exposed to the virus, but those that had were either dead or dying from pox disease. The ELISA test provided good evidence that grey squirrels were likely to be carrying the virus asymptomatically and passing it on to the reds with fatal consequences. Since then, studies of red and grey squirrels from across the UK have confirmed that pox disease in the red squirrels is only ever found in areas where the grey squirrels have been shown to have been infected with SQPV (Sainsbury *et al.*, 2000). To confirm that the virus was generally responsible for the disease observed in red squirrels and that grey squirrels remained unaffected, an experimental infection with virus was performed (Tompkins *et al.*, 2002). This indicated that grey squirrels did not generally suffer from disease, but that red squirrels developed an erythematous exudative dermatitis which progressed through haemorrhagic crusts to scabs. It was considered severe enough to suggest that red squirrels, infected in the wild, may not be expected to survive (Thomas *et al.*, 2003; Tompkins *et al.*, 2002) a suggestion that had previously been made (Sainsbury & Gurnell 1995; Sainsbury *et al.*, 1997).

The importance of SQPV in the decline of the native red squirrel was analysed comparing changes in red and grey squirrel sightings over time from areas where grey squirrels carried the disease (Norfolk and Cumbria) with areas where they did not (Scotland and Italy). The results suggested that in areas of interaction between

red and grey squirrels, where virus was present, the observed decline in the red squirrel population was 17-25 times more rapid than in areas where no virus was present (Rushton *et al.*, 2006).

In 2005, the first grey squirrels in Scotland observed to be seropositive for the poxvirus were detected just over the border from England. This was followed, two years later, by the first report of disease in Scottish red squirrels (McInnes *et al.*, 2009). Since red squirrels surviving the poxvirus infection in the wild are not normally found, the transmission of the virus from grey squirrels to red squirrels represents a clear risk to the survival of the red squirrel population in Scotland.

1.2 Poxviruses

Members of the *Poxviridae* family are amongst the most complex of known viruses. Members of the family cause important disease in humans, domestic animals and wildlife while others infect insects. Some are restricted in terms of the animal hosts in which they replicate and cause disease while others can infect more than one species. Some, while primarily causing disease in animals, are also zoonotic.

Until the discovery in 2003 of *Acanthamoeba polyphaga mimivirus* (APMV), another giant double-stranded dsDNA virus that grows in amoeba (Scola *et al.*, 2003), poxviruses were considered the largest known viruses (Buller & Palumbo, 1991). They have the unique property, which they share with other members of the proposed clade of large DNA viruses of eukaryotes (Hughes *et al.*, 2010) of completing their life cycle entirely in the cytoplasm of the host cell (Moss, 2007). As a result, poxviruses encode several proteins that are required specifically for their own replication outside the cell nucleus, as well as those that give them a replicative advantage in the face of the host immune response. A number of these proteins are secreted from the infected cell and are thought to interact with molecules produced as part of the host anti-viral response thereby subverting the natural host response to infection and increasing the survival of the virus.

There are several notable examples of the poxviruses. *Variola virus* (VARV), the agent which causes Smallpox disease, was reported to be the first virus to be seen

with a microscope (Buist, 1886) and *Vaccina virus* (VACV), another member of the *Orthopoxvirus* (OPV) genus, was the first animal virus shown to contain DNA but not RNA and to be visualized with electron microscopy (Fenner, 2000). *Myxoma virus*, a member of the *Leporipoxvirus* genus, was used as an effective biological control method for a vertebrate pest (for the control of the Australian wild rabbit) and in the process provided a unique natural experiment on the co-evolution of a virus and its host (Fenner, 2000).

Although poxviruses have been studied in the laboratory for many years, there is much still to be learned about their biological proprieties (Lefkowitz *et al.*, 2006). In this introduction to the poxviruses the general features of poxviruses will concentrate on the *Chordopoxvirinae* since there has been much more research performed on these in comparison to the other subfamily, the *Entomopoxvirinae*. Similarly, much of our current understanding comes from studies with *Vaccinia virus*, although where other viruses have been used this will be indicated in the text.

1.2.1 Taxonomic structure of Poxviridae

Members of the *Poxviridae* family can infect a large range of hosts. Within the animal kingdom the *Poxviridae* family is subdivided into two subfamilies; the *Chordopoxvirinae* (ChPV) which infect the vertebrates (ranging from avian species, reptiles, aquatic and terrestrial mammals) and the *Entomopoxvirinae* which infect insects. The *Chordopoxvirinae* is currently divided into eight genera with the names of the virus species that fall within these genera being presented in **Table 1.1**. A ninth genus, containing viruses that infect deer, has been proposed, the name of which would be the Cervidipoxvirus. Within a genus, the viruses are generally named according to the species infected or the disease caused (Fauquet *et al.*, 2005). Until now, the *orthopoxvirus* (OPV) and *leporipoxviruses* are the only two recognised genera which have members that cause disease in rodents.

Table 1.1 Poxviruses (modified from Robinson & Kerr, 2001)

Genus	Species
Avipoxvirus	*Fowlpox virus*[c]
Orthopoxvirus	*Buffalopox virus*[a], *camelpox virus, cowpox virus, ectromelia virus (mousepox), monkeypox virus, rabbitpox virus*[a], *raccoonpox virus,* skunkpox virus[b], *taterapox virus,* Uasin Gishu virus[b], horsepox virus[b], *vaccinia virus*[c], *variola virus (smallpox virus), volepox virus*, Steller sea lion poxvirus[b]
Parapoxvirus	Auzdyk virus[b], *bovine popular stomatitis virus,* chamois contagious ecthyma virus[b], *orf virus*[c], *pseudocowpox virus, red deer parapoxvirus,* seal parapoxvirus[b]
Cervidpoxvirus	Deerpox virus
Capripoxvirus	*Goatpox virus, lumpy skin disease virus, sheeppox virus*[c]
Leporipoxvirus	*Hare fibroma virus, myxoma virus*[c], *rabbit fibroma (*Shope fibroma*) virus,* squirrel fibroma virus[b]
Suipoxvirus	*Swinepox virus*[c]
Molluscipoxvirus	*Molluscum contagiosum virus*
Yatapoxvirus	*Tanapox virus, yaba monkey tumour virus*[c]
Unassigned	Cetacean pox virus, cotia virus, grey kangaroo pox virus, red kangaroo pox virus, quokka pox virus, molluscum-like viruses of horse, donkey and chimpanzee, mule deer pox virus, marmoset pox virus, **squirrelpox virus**

[a]Rabbitpox virus and buffalopox virus are regarded as variants of vaccinia virus
[b]Tentative members of the genus
[c]Prototypical virus

1.2.2 Virion morphology and structure

Although it is claimed that poxviruses are visible using the light microscope, knowledge of virion morphology comes from extensive studies with the electron microscope. Members of the P*oxviridae* family typically measure between 200 to 400 nm in length. The majority of poxvirions resemble orthopoxviruses in basic appearance; they have a characteristic enveloped brick shaped profile, covered with irregular tubular elements (**Figure 1.2**). Members of the same genus are

morphologically indistinguishable, but members of different genera often differ in size and slightly in morphology. The parapoxviruses (and squirrelpox virus) have a markedly different morphological appearance. They have a distinctly ovoid shape and the envelope's tubular elements are arranged more regularly, displaying a regular criss-cross spiral pattern (**Figure 1.3**) (Murphy, 1999; Fenner, 1994). As a result, electron microscopy (EM) can be useful as a differential diagnostic tool.

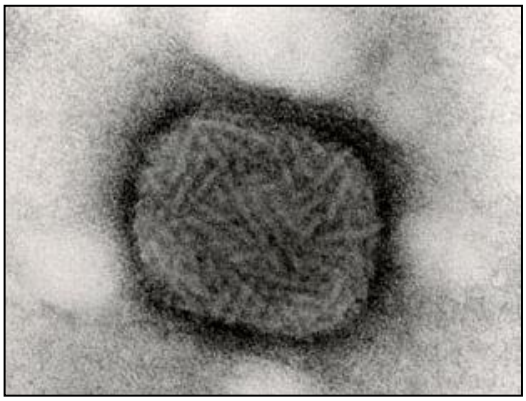


Figure 1.2 Variola virus particle.

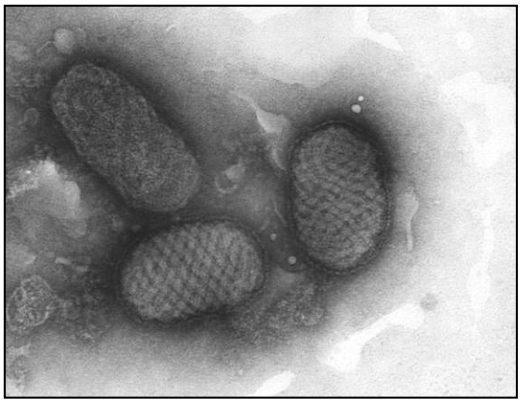


Figure 1.3 Parapoxvirus particles.

Negative staining transmission electron microscopy of two different genera belonging to the *Chordopoxviridae* family. Note the different morphology between the two virus genera.

The orthopox virion has more than 100 polypeptides arranged in four distinct structures; core, lateral body, membrane and envelope (**Figure 1.4**) (Buller & Palumbo 1991).

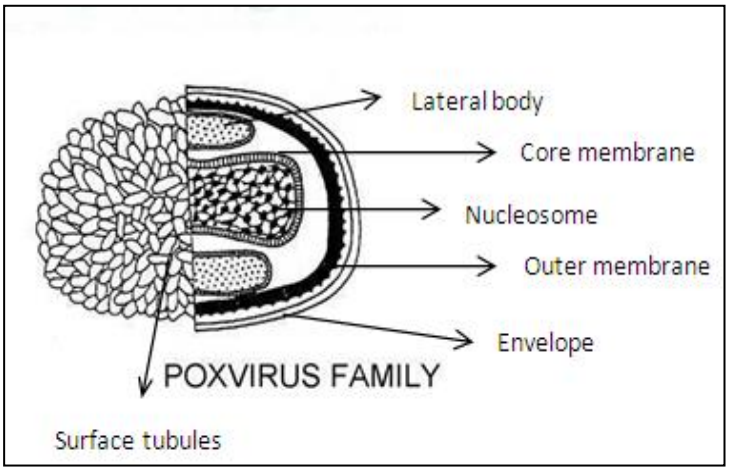


Figure 1.4 Schematic drawing of a typical orthopoxvirus (adapted from Murphy 1999).

Schematic ultra structures of *poxvirus* virion. On the left hand side: non enveloped structure showing the outer membrane covered with randomly arranged surface tubules. On a right hand side: section of enveloped virion.

1.2.3 Genome features of poxviruses

The poxvirus genome consists of a single molecule of linear double-stranded DNA; they range in size from approximately 130 kbp to 360 kbp in length, according to the species of virus, and typically encode 1 gene per kbp (Lefkowitz *et al.*, 2006). The terminal regions of the genome are covalently closed and possess inverted hairpin structures known as the inverted terminal repeats (ITRs) (Baroudy & Moss 1982; Fenner 1994) consisting of identical but oppositely orientated sequences of variable size (<0.1-12.4 Kb) (Lefkowitz *et al.*, 2006). The ITRs of the different OPVs varies in length due to various deletions, tandem repeat sequences and transposition (Wittek & Moss 1980; Esteban & Hutchinson 2011). Genetic analyses of poxvirus genomes that have been sequenced revealed that 88 genes are conserved in all chordopoxviruses (Mercer *et al.*, 2006) with about half of these also present in the entomopoxviruses (Gubser *et al.*, 2004; Upton *et al.*, 2003). These conserved genes

are usually essential for the survival and replication of the viruses and tend to be grouped in the central region of the genome, whilst genes that are non-essential for replication, but instead are involved in immunomodulation or host range are usually located in the more variable terminal regions (**Figure 1.5**) (Moss, 2007; Esteban & Hutchinson, 2011).

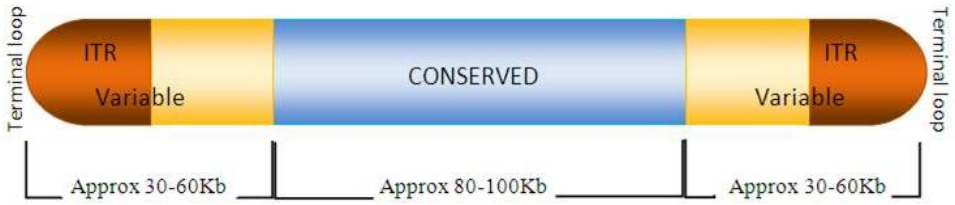


Figure 1.5 Diagrammatic representation of OPV genomes.

The conserved region among the OPVs is in blue with the variable regions ending with the tandem repeat (ITRs) on both sides.

In general poxvirus genomes are considered to have a base composition rich in A + T residues, the highest of which is *Sheeppox virus* (SPPV), a member of the *Capripoxviridae*, with 75% A + T residues (Xing *et al.*, 2006). There are exceptions to this with the *Parapoxviridae* and the *Molluscipoxviridae* having the opposite base composition skew. For example, *Orf virus*, the type species of the parapoxviruses, has approximately 64.5% G + C residues (Mercer *et al.*, 2006) and *Molluscum contagiosum virus* has a G + C content of 63% (Senkevich *et al.*, 1997). The squirrelpox virus genome has an even higher G + C content of ~66% (McInnes *et al.*, 2006).

Today more than 100 poxvirus genome sequences are available in Genbank, the vast majority of which are isolates of *Variola virus* (VARV) and *Vaccinia virus* (VACV). However, since VACV Copenhagen isolate was the first poxvirus genome to be sequenced in its entirety, it is often used as the reference genome with which comparisons are made.

1.2.4 Poxvirus replication

All poxviruses replicate exclusively in the cytoplasm of the infected cell. The replication cycle has been studied using vaccinia virus, but the essential features are highly conserved amongst other poxviruses (Moss, 2007).

Replication is a complicated sequence of events that begins with binding of the virion to the cell surface, subsequent fusion of the virus and host cell membrane and entry into the cell. Poxvirus entry is followed by the expression of virus early genes and uncoating, replication of the virus genome and expression of late genes, morphogenesis of new virus particles and egress from the cell (**Figure 1.6**).

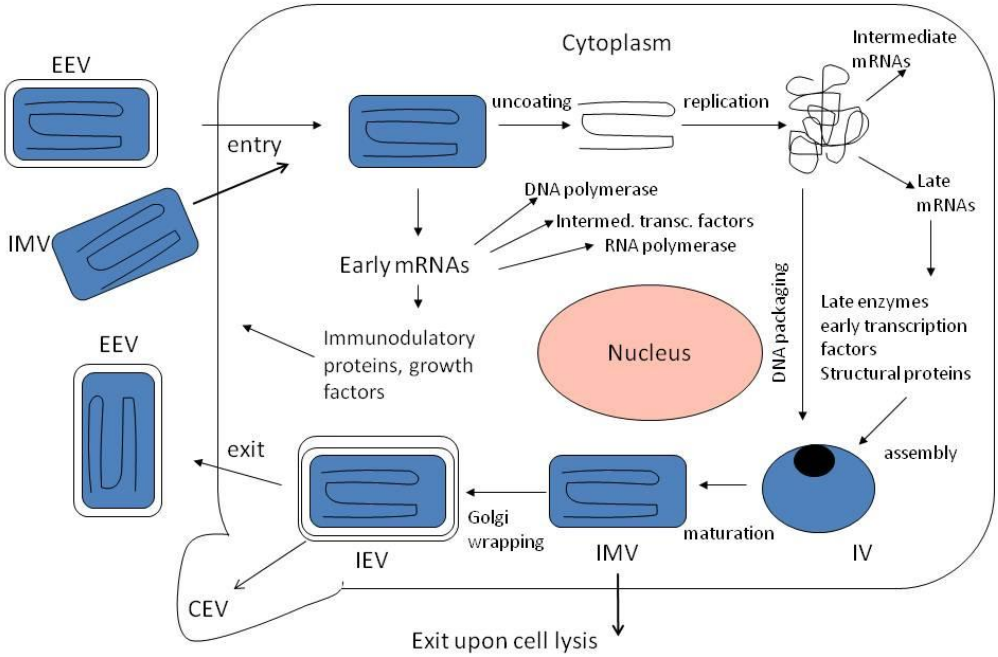


Figure 1.6 Schematic representation of the replication cycle of a poxvirus (Adapted from Moss, 2007).

The morphologically distinct forms, the intracellular mature virus (IMV), the extracellular enveloped virus (EEV) or the cell-associated enveloped virus (CEV) can initiate infection. After the virus entry, different stages take place which result in the production of immature viruses (IV) and their maturation to new mature virus (IMV). A portion of IMV is transported to the Golgi apparatus where it becomes enveloped by two additional lipid membranes (IEV). The vast majority of IMV are released upon cell lysis. IEV are transported by microtubules to the cell surface where they can remain as cell-associated viruses (CEV). A small proportion of these lose their outer membrane by fusing with the cell membrane thereby allowing egress of the EEV.

Both intracellular mature virus (IMV) and extracellular virions, that include both the cell-associated enveloped virus (CEV) and the extracellular enveloped virus (EEV), can initiate the infectious cycle (Robinson & Kerr, 2001; McFadden, 2005). The exact mechanism by which the virus enters into the cell is still the subject of investigation. Recently a study has shown that the route of virus entry can depend on different viral proteins, with the determinant of binding being regulated by different glycosaminoglycans present at the host cell surface or other components of the extracellular matrix (Bengali *et al*., 2009). Other studies have shown that plasma membrane fusion is accelerated by acid treatment and that entry has many characteristics of macropinocytosis (Mercer & Helenius, 2008). Although no specific cell receptors are known to be required for virion fusion and entry, there is evidence that this mechanism can be associated with several host protein-kinase signalling pathways.

The internalization of viral particles in the host cell results in the removal of the lipidic membranes from the EEV and IMV with the release of viral cores in the cell cytoplasm. Poxvirus replication takes place in the areas of the cytoplasm called factories or the viroplasm. These areas react with basophilic stains and appear, when observed with light microscopy, as structures which are referred to as B-type inclusion bodies or Guarnieri bodies (Kato & Cutting, 1959).

Once the virus cores accumulate in the perinuclear region of the cell, partial uncoating occurs and the virus genome begins to be transcribed by the virus-encoded-RNA polymerase beginning with the early viral genes (Roberts & Smith, 2008) (**Figure. 1.6**). This limited expression of the infecting genome is thought to be required for the next stage known as uncoating, which induces the complete dissolution of the core structure (McFadden, 2005).

Early transcription is believed to be exclusively under the control of virus-encoded transcription factors, which are encapsidated within the core. Early gene products are generally proteins essential for DNA replication, intermediate gene transcription and host immune modulation (Moss, 2007) which allow the virus to escape from the host innate immune response and facilitate the virus DNA replication. The uncoating step

releases the viral DNA where it can function as template for DNA replication and the subsequent steps of intermediate and late gene transcription. Completion of the uncoating is described as the conversion point at which the poxvirus becomes susceptible to the action of DNase (Joklik & Becker, 1964). DNA replication appears to occur at the same time or just prior to intermediate gene expression and is mediated by the virus-encoded DNA polymerase.

The subsequent transcription stages require host-derived transcription factors, in addition to further virus-encoded factors (Broyles *et al.*, 1999). Intermediate expressed genes tend to encode the late transcription factors, whereas the late expressed genes encode virion structural proteins as well as early transcription factors and other proteins that are encapsidated within the infectious virus particle (Broyles *et al.*, 1999).

Production of progeny virus, as it's schematically shown in **Figure 1.6**, starts after DNA replication and as the late viral gene products are accumulating within the cell. Viral morphogenesis is a process during which the viral genome is assembled together with viral proteins into a nucleoprotein complex which forms part of the core. This is then wrapped in a membrane to form a virion particle which then matures through various steps to become the infectious particles. These intracellular mature virions (IMVs) can become wrapped in extra Golgi-derived membranes to form intracellular enveloped virus (IEV). Migration of IEV to the membrane of the cell appears to be mediated by the microtubule network whereupon the IEV form loses one of its outer membranes as it fuses with the cell membrane to form the cell-associated enveloped virus (CEV) which can potentially infect neighbouring cells by actin-tail polymerization, or are released directly as free extracellular enveloped virus (EEV) particles.

1.2.5 Immunopathogenesis of poxvirus infection

It has been suggested that whilst smaller viruses may subvert the immune response of the host by genomic drift or by being able to replicate rapidly, the larger viruses need a more complex strategy to survive in the host (Stanford *et al.*, 2007).

Humoral and cell-mediated immunity both have an important role in virus clearance and to the protection of the host from reinfection. For the vast majority of poxviruses

the antibody response is measurable within a few days. The antibodies can control infection through different mechanisms; virus neutralization, complement activation, opsonisation and antibody-dependent cell-mediated cytoxicity (ADCC) (Smith & Kotwal, 2002). However, the role of antibody in protecting the host from a primary infection differs depending on the virus, the host, the route of inoculation and the infectious dose. For example, despite the presence of antibodies, sheep are not protected from infection with *Orf virus* whereas antibodies do appear to be responsible for long lasting immunity to *Variola virus* (Smith & Kotwal, 2002).

In poxvirus infections, both the innate and the acquired immune response are important. The innate immune response is an antigen non-specific response characterized by the induction of apoptosis, the activation of complement, macrophages and natural killer cells (NK) and by the production of interferons (IFNs) and inflammatory cytokines. The innate immune response acts in the first stage of infection while the adaptive (acquired) immune response is antigen-specific and generally follows a few days after the innate response (Smith & Kotwal, 2002). The virus' ability to subvert the innate response is predicted to interfere with the ability of the host to mount a successful adaptive response to infection therefore ensuring a successful infection (Haig, 2001). NK cells form part of the innate immune response and are involved in NK cell-mediated cytotoxicity and production of cytokines. Their capacity to begin the lysis of virus infected cells, probably due to the reduction of MHC class I molecules on the infected cell surface, comes before cytolytic T cell (CTL) mediated killing. However, they alone cannot control the spread and development of disease (Smith & Kotwal, 2002).

Poxvirus-specific CTLs are thought to play one of the most important roles in controlling poxvirus infections, resulting ultimately in the clearance of the infection (Smith & Kotwal, 2002). Poxvirus antigen-specific CTLs are generated within few days of infection and are able to recognize and eliminate the infected cells. As a consequence, the viruses have evolved several strategies to avoid the host immune defence reducing the efficacy of the cell-mediated immunity (CMI) response (Seet *et al.*, 2003). One of these mechanisms is to reduce the expression on the infected cells of molecules that participate in antigen presentation and immune cell recognition. The ability of poxviruses to inhibit pro-inflammatory cytokines such TNF and IFN

that regulate MHC expression, provide one of the aspects of down-regulation of MHC class I on the infected cell surface. Some poxviruses also express their own version of MHC class I molecules which appear to down regulate expression of the endogenous MHC molecules at the cell surface. Poxviruses can also down regulate the expression of other cell surface molecules which are important in the host immune response; indeed infection with myxoma virus can cause down-regulation of CD4 expression (Seet *et al.*, 2003).

Poxviruses have been shown to encode several proteins which play an important role in evading and modulating the host immune response and, in the process, determine the virus virulence (Seet *et al.*, 2003; McFadden, 2005; Alcami, 2003). These immunomodulatory mechanisms have primarily been elucidated over many years of studying VACV and the other orthopoxviruses, but even before many of the full genome sequences became available it was apparent that the different genera, and maybe even species of poxvirus, possessed different, and sometimes unique, ways of subverting the host immune response. To date, immunomodulatory genes have been identified from many viruses including the orthopoxviruses, *molluscum contagiosum virus* (MOCV), the capripoxviruses, the parapoxviruses and the leporipoxviruses, with *Myxoma virus* (MYXV) receiving most attention within that genus. The ways in which poxviruses are now known to interact with the host immune response to infection are so numerous that a full review of all the mechanisms used by the different viruses is not possible here. Equally, since many of the host anti-viral pathways are inter-connected only a few examples will be highlighted.

Modulator proteins produced by poxviruses collectively modulate a wide range of antiviral defence responses that are triggered by the virus infection and which include important host pathways such apoptosis, interferon induction, stress-induced signalling cascades, MHC-restricted antigen presentation and pro-inflammatory pathways (Seet *et al.*, 2003). These modulatory proteins include: complement control protein (VCP), double-stranded-RNA binding proteins, cell growth factors, cytokines and receptor mimics for chemokines and cytokines such as interlukin-1, tumor necrosis factor and type 1 interferons (Smith & Kotwal, 2002), amongst others. Other proteins are able to down regulate the expression of important host molecules by interfering with cell signalling pathways.

The absence of complete conservation of the virus-encoded immunomodulatory genes between different poxviruses may explain the differences observed in the host and tissue tropism displayed by the different viruses. However, despite the lack of absolute conservation, the host immune pathways that are consistently targeted by all poxviruses include those related with the production of interferons and chemokines (Seet *et al.*, 2003).

Three type of interferon (α, β, γ) are produced by virus infected cells to control poxvirus growth. The actions of the interferons result in the activation of macrophages, NK cells, and cytotoxic T-lymphocytes and also in the induction of an antiviral state in neighbouring un-infected cells through the production of interferon-inducible genes. It is maybe for this reason that many of the genes encoded by poxviruses target the IFN response in particular.

1.2.6 The role of the host in poxvirus pathogenesis: host tropism

The host range and lethality of poxvirus infection varies considerably. Some can infect a wide range of animals and can be zoonotic while others have a very limited host range. Productive infection is defined by the replication and transmission of the virus. The outcome of infection is a function of both the infectious agent and the host response to it. The tendency for immunomodulatory genes to be conserved within a genus, but less well conserved between different genera may reflect differences in host and tissue tropism and may explain the phenotypic difference observed during clinical manifestations of poxviral disease (Seet *et al.*, 2003), but the capacity of the host to mobilize the immune defences also plays an important role in the course of disease and its severity (Stanford *et al.*, 2007).

McFadden (McFadden, 2005) has defined three levels of poxvirus tropism; the first is cellular tropism, which considers the capacity of a virus to replicate in different cell types; the second considers the capacity of a virus to replicate in specific organs or tissues. Crucial to this are the factors that influence cellular tropism and the innate antiviral capacity of particular tissues. The third level, takes into consideration the other two levels in combination with the overall host immune and inflammatory response. This level describes the pathogenesis, where signs of disease occur in the infected organism. The second and third levels influence the primary and subsequent

sites of replication within a particular host and influence transmission of the virus. It is considered that in a reservoir host virus can be transmitted while being generally of low pathogenicity and often only causing subclinical infection (McFadden, 2005).

In the case of SQPV the same virus can infect both species of squirrels but causes disease only in the red squirrel. In the reservoir host (grey squirrel), the virus may have evolved with the host and become less virulent, as has been recognised for *Myxoma virus*, a member of the *leporipoxvirus* genus (Kerr & McFadden, 2002). *Myxoma virus* can infect the European rabbit (*Oryctolagus cuniculus*) under natural conditions, causing a systemic disease that may be lethal, but it causes a relatively benign disease in the South American rabbit (*Sylvilagus brasiliensis*).

1.2.7 Transmission of poxviruses

Several routes of poxvirus infection have been identified, some natural and some as a result of experimental infection. Specifically, poxviruses are known to infect the host animal *via* the respiratory tract, through broken or abraded skin or mucosa or by percutaneous inoculation by arthropods. Infection *via* the respiratory tract has been best documented for VARV, VACV and *Ectromelia virus* (ECTV) although, in the case of ECTV infection, it is not known how important this is to the natural infection.

Ones of the commonest routes of infection used by poxviruses is the introduction of virus particles into small skin abrasions directly by skin to skin contact or indirectly through environmental contamination with the virus (Murphy, 1999). The scabs that are often shed as a result of a poxvirus infection are heavily laden with virus and given the right environmental conditions the virus can survive in dried scabs or other material such as bedding, for many years (Downie & Dumbell, 1947; Essbauer *et al.*, 2007). Indeed, natural infection with ECTV is thought to occur *via* minor abrasions in the skin, through contaminated bedding or during manipulations by animal handlers in laboratories (Fenner 1949). The oral route has also been suggested in ECTV infection, but it is possible that this occurs through damage to the oral mucosa. The oral route is not thought to be of major importance in natural infection, but when it does occur, virus is shed in the faeces for a long period (Wallace & Buller, 1985).

It is important to note that infection with the virus through the skin normally results in a localized infection, near the site of entry, usually in the epidermis and dermis, which

can lead to a systemic infection and generalized skin lesions, although not always. The same virus can cause a localized infection in one host and generalized disease in another, even if the hosts belonged to the same family but different animal species. The different aspects and outcome of disease generally depend on the virus strain, the immunological state of the host and its genetic makeup (Stanford *et al*., 2007), even when the infection has been acquired *via* the same route. *Cowpox virus* (CPXV), for example, can cause both localized and generalized lesions in domestic animals and humans. Several cases of CPXV in humans presenting with skin lesions due to scratches, contact or bite from infected animals are reported (Postma *et al*., 1991; Coras *et al*., 2005; Campe *et al*., 2009; Hawranek *et al*., 2003; Nitsche *et al*., 2007) and some resulted in viremia (Nitsche *et al*., 2007) or even in a fatal disease (Eis-Hubinger *et al*., 1990). Similarly, a veterinarian, who suffered from psoriasis and was in contact with a CPXV-infected cat, suffered multiple lesions on his hands and face (Lawn, 2010). CPXV manifests generally as focal skin lesions in domestic cats, but systemic diseases in felids are reported (Kurth *et al*., 2009; Kaysser *et al*., 2010)

Arthropods play an important role as mechanical vectors in poxvirus transmission. The viruses causing sheeppox, goatpox, lumpy skin disease, myxomatosis, fibromatosis and fowlpox have all been documented as being transmitted mechanically by biting arthropods. In experiments it has been possible to transmit the squirrel fibroma virus by mosquitoes *Aedes aegypti* and *Anopheles quadrimaculata* (Kilham & Dalmat, 1955), with suckling squirrels being most susceptible. Terrell *et al*., (2002) described an epidemic of squirrel fibromatosis, which was associated with large numbers of fleas, and concluded that natural infections with squirrel fibroma virus are most likely to be transmitted by biting arthropods such as the squirrel flea (Robinson & Kerr, 2001).

With SQPV, nothing is known about the natural route of infection or the means of transmission between individual animals. Rushton *et al*., (2000) have proposed several routes for transmission. Direct transmission from the environment via dreys known to be used by both species or at scent-marking sites within the same home range were considered possible routes. Red squirrels have scent glands on the upper and lower lips and leave marks by face-wiping behaviour on branches and roots, but also by anal-dragging. In this context, Rushton *et al*., (2000) noted that the lesions

on red squirrels were in areas which might be scratched through scent-marking on bark.

1.3 Poxvirus infection in lagomorphs and rodents

In the last few decades more attention has been paid to the role of wildlife as reservoir hosts for several infections and emerging diseases. Relatively little is known about natural poxvirus infections of wild animals. Many poxviruses and their wildlife hosts seem extremely well adapted to each other. Infection is often only detected when they spill over from their natural host into a susceptible host causing disease. Some the best known examples are listed below.

1.3.1 Myxoma virus

Myxoma virus (MYXV) is the etiologic agent of myxomatosis in rabbits. Under natural conditions, MYXV appears to have a narrow host range. The American bush rabbit (*Sylvilagus bachami*) and the tapeti (*Sylvilagus brasiliensis*) are considered the reservoir species of two different strains of MYXV, the first for a strain found in the western portion of North America and the second for the South American strain (Fenner & Ratcliffe, 1965). These wild American rabbits are generally more resistant to the effects of the virus. Whilst they may become infected, showing a localized benign cutaneous fibroma, and serve to spread the disease, they generally do not suffer from a high mortality rate. In contrast, the virus produces recognizable lesions and generalized systemic disease in the domestic or European rabbit (*Oryctolagus cuniculus*). No other animal species is known to be susceptible to *Myxoma virus* infection.

The American strain of MYXV, originating from and maintained in the South American forest rabbit population, was deliberately released in Australia in 1950 as a rabbit demographic control measure to reduce feral European rabbit populations. The same strategy was also adopted in Europe. After an initial dramatic decimation of the rabbit population, the mortality rate dropped. Myxoma virus now is endemic across Europe and Australia. Control of the rabbit population has failed due to genetic changes of the virus and adaptation of surviving rabbits. With the passage of time

attenuated virus has been isolated from wild populations of the domestic rabbit. Inoculation of this strain into laboratory rabbits has shown that virus has deceased in virulence by at least a factor of five (Buller & Palumbo, 1991).

The incomplete success in Australia has also been attributed to climatic factors that correlate with the presence of a vector for the spread of the virus. Whereas in temperate regions the virus still affects up to 60% of feral rabbits, despite some rabbits having developed limited resistance to the virus, a lack of mosquitoes in semi-arid regions of Australia has inhibited the spread of myxomatosis. An arid climate-adapted rabbit flea has now been introduced into Australia to assist with the spread of myxomatosis (Burley, 1986).

1.3.2 Shope fibroma virus

Like MYXV, *Shope fibroma virus* (SFV) belongs to the *Leporipoxvirus* genus, a family of viruses whose host range includes rabbits, hares, and squirrels. Fibroma virus infection was first described by Shope in 1932 as a naturally occurring disease in eastern cottontail rabbits (*Sylvilagus floridanus*). It causes a fibroma reminiscent of those caused by MYXV in its natural hosts and as far as is known does not cause the generalized disease associated with MYXV. The virus that was able to cause a fibroma was experimentally transmitted to domestic rabbits (*Oryctolagus cuniculus)*. Healthy adult rabbits mount an effective cell-mediated immune response, which typically starts to reduce tumor growth within 10±12 days post infection (Scott *et al*., 1981b; Sell & Scott, 1981).

The Leporipoxviruses appear to be transmitted between rabbits by biting insects, and the widespread prevalence of antibodies to the virus suggests that SFV infections may be endemic throughout the US and Canadian rabbit populations (Digiacomo & Mare', 1994). DNA sequence analyses have confirmed that SFV is closely related to MYXV.

1.3.3 Cowpox virus

Edward Jenner was the first to notice that milkmaids who had been in direct contact with cowpox infected cattle and who had localized lesions on their hands were protected against smallpox infection (Burton, 1994). Originally it was suggested that vaccinia virus may have arisen as a genetic hybrid between variola major and

cowpox virus but subsequent studies have demonstrated that cowpox virus is distinct from vaccina virus (Downie & Dumbell, 1956) and is now known to be distinct from all other orthopoxviruses.

Cowpox virus (CPXV) belongs to the *Orthopoxviridae* genera within the *Poxviridae* family (Fauquet *et al*., 2005) CPXV is still occasionally seen in milking cows with even rarer zoonotic transmission to the hands of milkers through contact with pocks on the teats (on occasion it can be confused with milker's nodule disease which is caused by a parapoxvirus, namely *Pseudocowpox virus*). Human cowpox was regarded as an occupational disease of dairy farm workers (Baxby, 1977a), although with the advent of automated dairying systems this source of infection has been much reduced. Despite its name, cattle are not the main host of CPXV. It is well known to infect a broad range of mammals. The virus is able to cross the species barrier, apparently more so than most other poxviruses, and can cause death in a large variety of mammals species, from wild and domestic felids, to elephants and primates (Baxby *et al*., 1979; Baxby *et al*., 1982; Bennett *et al*., 1986; Essbauer *et al*., 2010; Hemmer *et al*., 2010; Marennikova *et al*., 1977; Schmiedeknecht *et al*., 2010).

Geographically, CPXV is endemic in northern Europe and western and central Asia (Bennett & Baxby, 1996; Essbauer *et al*., 2007). Several biotypes are in circulation and it is argued that the survival of these biotypes is required for enzootic infections in some species (Baxby, 1977b). Since CPXV is not particularly resistant and would not be expected to survive for long in the soil, it was argued that cattle could not be the natural host and reservoir of "cowpox" but instead became infected accidentally, from another host, mostly likely to be a small wild mammal (Baxby, 1977a). It is now well accepted, that wild rodents are the reservoir and maintenance hosts of CPXV (Essbauer *et al*., 2010; Hazel *et al*., 2000; Chantrey *et al*., 1999; Baxby, 1977a). In Great Britain antibodies against cowpox have been found with highest prevalence in bank voles (*Myodes glareolus*), field voles (*Microtus agrestis*) and wood mice (*Apodemus sylvaticus*) with viral DNA also being confirmed in these species by polymerase chain reaction (PCR) (Chantrey *et al*., 1999). Indeed it is suggested that these species are the main virus hosts in the UK and much of Europe (Chantrey *et al*., 1999), while wild rats (*Rattus norvegicus*), and domestic animals,

such as cattle and cats, are more likely to be incidental hosts. Evidence of the virus has also been found in ground squirrels (*Spermophilus citellus fulvis*), and gerbils (*Rhombomys opimus*in) in Turkmenistan and Georgia (Baxby, 1977a; Marennikova *et al*., 1984; Tsanava *et al*., 1989), in root voles in northern Russia and by PCR in various rodents in Norway (Tryland *et al*., 1998). The status of these animals as natural hosts remains unclear.

Cats as predators are exposed to CPXV while hunting rodents which serve as a reservoir for CPXV (Pfeffer *et al*., 2002). In felids, the outcome of the infection seems to depend on the CPXV strain, route and site of administration and most likely the dose of infection (Essbauer *et al*., 2010). Even if cats are considered the commonest examples of pets infected by CPXV (Martland *et al*., 1985; Kaysser *et al*., 2010), recently CPXV was also diagnosed in a dog with a focal nodule on the muzzle where it is thought that the virus had entered through abrasions on the skin (von Bomhard *et al*., 2011).

1.3.4 Monkeypoxvirus

Monkeypox virus (MPXV) is another orthopoxvirus (Fauquet *et al*., 2005). The virus was named after it was first recognized in association with outbreaks of vesicular exanthemas among captive primates in laboratories and zoos during the 1950s and 1960s (Arita & Henderson, 1968; Arita *et al*., 1972). Historically, the first recognition of monkeypox as a specific entity in the group of poxvirus diseases was recorded by von Magnus and colleagues in 1959 (Arita & Henderson, 1968) when they first isolated the virus in 31 Cynomolgus monkeys (*Macaca fascicularis*) from Singapore.

Since the global eradication of *Variola virus* (VARV), the causative agent of human smallpox, in 1977, MPXV has emerged as the most significant public health threat from the poxviruses. Unlike VARV, which was a pathogen exclusive to humans, MPXV has a broad range of hosts; outbreaks of disease have been documented in the Old and New world in wild and captive mammals respectively, including monkeys, apes and rodents such as rats, mice, squirrels, prairie dogs and rabbits (Spickler & Roth, 2008). Despite the virus name, human and non human primates are thought to be accidental hosts and infections are hypothesized to result from contact with an

infected sylvan animal, where the principal reservoirs are thought to be squirrels and other rodents (Greenwood, 2003). The natural cycle of MPXV within its native ranges is still poorly understood, e.g. its route of transmission to humans in the tropical forest setting (Reynolds *et al*., 2010), although it has been suggested that virus may circulate among arboreal mammals, mainly squirrels (Khodakevich *et al*., 1987).

The first MPXV isolated from the wild was obtained from a single squirrel (*Funisciurus anerythrus*) with generalized skin lesions. The animal was captured moribund during a survey investigation subsequent to a previous human outbreak in Zaire (Khodakevich *et al*., 1986).

Recently, the presence of clinical illness in human patients in the USA was associated with MPXV-infected prairie dogs (*Cynomys* sp.). These rodents had become sick after being housed in the same pet stores as exotic African rodents (Robert *et al*., 2004; Langohr *et al*., 2004). Laboratory tests also demonstrated the presence of monkeypox virus in several other rodents (*Cricetomys* sp. and *Graphiurus* sp.) that had died unexpectedly but without exhibiting the characteristic signs of monkeypox; these rodents had originated from the same shipment of prairie dogs responsible for the human cases (Langohr *et al*., 2004; CDC 2003). As a result of this outbreak of disease and the similarity between monkeypox and smallpox (Khodakevich *et al*., 1988), several experimental infections have been performed using ground squirrels (*Spermophilus tridecemlineatus and Cymonis* sp.) as animal models for studying the pathogenesis and virus transmission and for devising new vaccines and treatments for monkeypox (Hutson *et al*., 2009; Sbrana *et al*., 2007; Robert *et al*., 2004; Guarner *et al*., 2004; Langohr *et al*., 2004).

1.3.5 Squirrel fibroma virus

Squirrel fibromatosis (SF) is a cutaneous disease which results in individual or multiple cutaneous tumours (Carlton & Reilly, 1955; Kilham *et al*., 1953; Robinson & Kerr, 2001) and has been reported frequently in tree squirrels. The disease is caused by a leporipoxvirus which is related to rabbit fibroma virus (Fenner, 1965). The first suspected case of SF in a grey squirrel was reported in Maryland, USA (Kilham *et al*., 1953).

Naturally occurring SF predominantly affects grey squirrels and occurs mainly along the USA's eastern seaboard including Florida, Maryland, North Carolina, West Virginia and up to Connecticut (Terrel *et al*., 2002; King *et al*., 1972; Hirth *et al*., 1969). It is reported less frequently in western US but does occur in Ontario, Canada (Regnery, 1975). Recently, cases SF have been reported in American red squirrels (*Tamiasciurus hudsonicus*) from northern Indiana (Bangari *et al*., 2009) and a suspected case from north-western Canada (Himsworth *et al*., 2009).

Squirrel fibromatosis is considered to be a sporadic disease, although epizootics have been reported in grey squirrels in Florida (Terrel *et al*., 2002). The normal pathogenesis of the disease is that the cutaneous tumours regress spontaneously similar to what occurs with rabbit fibroma virus (Kilham *et al*., 1953; O'Connor *et al*., 1980). However, the development of large tumours leading to severe tissue damage and secondary complications does occur and there are reports of cases of naturally occurring disease in eastern grey squirrels and the American red squirrels, where virus was in found also in internal organs such as lungs, liver and kidney (King *et al*., 1972; Bangari *et al*., 2009). Some data suggests that viral transmission may be associated with biting arthropods, including mosquitoes and the squirrel flea (*Orchopeus howardi*) (Bangari *et al*., 2009), which act as mechanical vectors as is well documented for the transmission of MYXV.

Although the complete genomic sequence of the causative virus of SF has not been reported, the squirrel fibroma virus (SQFV) has been tentatively classified as a member of the leporipoxvirus genus according to the gross and histological lesions that occur during the course of clinical disease, the immunological cross-reactivity with rabbit fibroma virus and its ultrastructure when viewed by electron microscopy. Recent molecular studies of SQFV isolated from both grey and red squirrels from northern Indiana have confirmed the close correlation of SQFV with the members of leporipoxvirus genus, particularly Shope fibroma virus (Bangari *et al*., 2009).

Squirrel fibromatosis is diagnosed clinically by the presence of dermal nodules and the characteristic histological changes associated with the lesion. However, a definitive diagnosis of cutaneous fibroma poxvirus-associated disease can only be achieved by the use of molecular tools such as a specific PCR assay. Grossly, the

infection is characterised by single or multiple fibro-cutaneous nodules ranging from 0.2-2.5cm in diameter which are frequently alopecic and located primarily on the head and limbs, especially at muco-cutaneous junctions including the perianal region (King *et al*., 1972). Histologically, lesions are typical of poxviral infections; epidermal hyperplasia, ballooning degeneration of the keratinocytes of the stratum spinosum and the presence of intra-cytoplasmic inclusion bodies (Bangari *et al*., 2009). A characteristic specific to lesions of SF is proliferation of atypical mesenchymal fibroblasts in the dermis which, along with the keratinocytes, contain intra-cytoplasmic inclusion bodies (Hirth *et al*., 1969; King *et al*., 1972; Terrel *et al*., 2002).

Systemic disease with lesions including the lungs, liver and kidneys has also been reported in experimental infections (Kirschstein *et al*., 1958). When SF manifests as a systemic disease, the lung contains adenomatous nodules derived from proliferation of alveolar epithelial cells which frequently contain eosinophilic cytoplasmic inclusion bodies (King *et al*., 1972; Bangari *et al*., 2009). Nodules present in the liver are comprised of proliferations of atypical mesenchymal loose connective tissue in the portal areas. These lesions are similar to those present around the bronchioles in the lungs and in the dermis (King *et al*., 1972). Experimental transmission of SQFV and viral replication is possible in woodchucks (*Marmota monax*) (Robinson & Kerr, 2001). Domestic rabbits (*Oryctolagus* sp.) have been also experimentally challenged with SQFV, resulting in a dermal reaction at the site of injection within 3 days and followed by regression of the lesion by 11 days post-challenge (Hirth *et al*., 1969).

1.3.6 Squirrelpox virus

Squirrelpox virus (SQPV) infection has been fatal to red squirrels in Great Britain and Ireland (*Sciurus vulgaris*) and with the exception of the one case reported by Duff *et al*., (1996), there is no evidence of clinical disease in grey squirrels even if most grey squirrels tested in the UK have been seropositive for SQPV (Sainsbury *et al*., 2000). The origin of SQPV is still unknown, although it is considered that grey squirrels imported from the USA are asymptomatic carriers of the virus which is now spreading within the UK, contributing the decline of the native red squirrels. Although SQPV has never been isolated from grey squirrels in the USA, serological

samples collected from North America have tested positive for antibodies against SQPV (McInnes *et al*., 2006).

On the basis of post-mortem findings and histological examination in red squirrels, SQPV shares features with other members of poxviridae. The clinical signs of the disease resemble those of the other poxviruses; cutaneous lesions are commonly located around the thinly haired areas of the head, pinna, digital and ventral skin. The typical histopathological lesions showing hyperkeratosis of the epidermis, ballooning degeneration of keratinocytes and formation of intracytoplasmic inclusion bodies in the keratinocytes. The macroscopic lesions induced by SQPV are characterised by erythematous exudative dermatitis and ulceration with some lesions covered by haemorrhagic scabs (Tompkins *et al*., 2002; Scott *et al*., 1981a). SQPV is the sole member of an unclassified genus which seems to be genetically related to the *Parapoxvirus* genus and *Molluscum contagiousum virus* genus (McInnes *et al*., 2006). Little is known about its epidemiology. Recent epidemiology studies on SQPV have shown that seroprevalence in grey squirrels increases during the autumn and spring and is more associated with adult males (Bruemmer *et al*., 2010). This could suggest that the infection is acquired during the mating and dispersal period when males expand their home range and there is more physical contact and sharing of space between red and grey squirrels.

1.4 Aim of the thesis

The past years have seen the decline of the red squirrel (*Sciurus vulgaris*) population in the UK. Squirrelpox virus (SQPV) has had a significant impact on this decline and it is thought to be the major factor in competition between the native red squirrel and the introduced North American grey squirrel (*Sciurus carolinensis*) (Rushton *et al*., 2006). The grey squirrel has a high seroprevalence to SQPV in England and Wales and it is thought to be the reservoir host for the virus and to transmit the infection to the red squirrel (Sainsbury *et al*., 2000). Furthermore, experimental infection studies have confirmed that both grey and red squirrels can be infected with the virus (Thomas *et al*., 2003; Tompkins *et al*., 2002). However, whilst the grey

squirrel does not show any apparent clinical signs of the disease, red squirrels suffer disease severe enough to cause high mortality in the wild (Tompkins *et al*., 2002).

At the present nothing is known about the transmission of SQPV between grey squirrels and from grey to red squirrels, there is therefore a need to investigate the transmission dynamics and difference in pathogenicity between two species.

The aim of this thesis is to further investigate the contention that grey squirrels are acting as a reservoir for the SQPV. It is unknown if the virus replicates in grey squirrels and, if it does, in which organs/tissues this replication takes place. It is further unknown how the virus is transmitted from one grey squirrel to another or indeed from grey to red squirrels.

This thesis will investigate the potential mode of SQPV dissemination by examining virus presence in tissue samples from infected red and grey squirrels. Particular emphasis will be given to an experimental infection of grey squirrels with SQPV to derive a better understanding of the mechanism associated with the infection in that species. Several techniques will be employed throughout the study to demonstrate the course of infection in grey squirrels, in particular: post mortem analysis, molecular biology assays (PCR and qPCR), serology test (ELISA), histopathology and immunohistochemistry. Aspects of molecular epidemiology will be also considered in order to better understand the evolution of SQPV, including analysis of different virus isolates collected over the course of the last 12 years from different outbreaks across the UK.

CHAPTER 2

MATERIALS AND METHODS

2.1 Centrifuges and Common reagents

Where not specified differently, all the centrifugation steps were performed in a bench top microcentrifuge, model 1-15P (Sigma-Aldrich).

For a full list of reagents see **Appendix 1.0**

2.2 Sample storage

Clinical samples collected during post mortem examination of grey squirrels experimentally infected with squirrelpox virus (SQPV) (**Chapter 4** and **Chapter 5**) were stored at -70°C prior to DNA extraction. Samples obtained from previous experiments and from naturally infected red squirrels were also stored at -70°C until required. These included scab and tissue samples from SQPV infected red squirrels used for the preparation of virus inocula. Carcasses of red squirrels submitted to Edinburgh University School of Veterinary Medicine (**Chapter 3**), were frozen at -20°C if post mortem examination was not performed on the same day. Red squirrel tissue samples collected during the post mortem examination were stored at -20°C in first instance and subsequently at -70°C for long term storage. Stocks of SQPV scabs, virus inocula and virus purified from cell cultures were also stored at -70°C. Nucleic acids were stored at -20°C.

2.3 Virology

2.3.1 Cell culture

2.3.1.1 Production of cultured cells for growth of SQPV

Foetal lamb skin cells (FLSc 1830/2/3/2 or 1830/2/3/3) were resuscitated from storage in liquid nitrogen for growth and titration of squirrelpox tissue culture adapted virus (Red Squirrels SPPV 230, FLM 19, 10 Feb 1999). These cells were

also used for the growth of SQPV directly from scabs obtained from naturally SQPV infected red squirrels (§**2.3.1.2**).

The vial containing the cells (1 ml tube with $5x10^6$ cells, stored in liquid nitrogen), was placed in a water bath at 37°C and the cells defrosted gently. It was removed from the bath before being completely defrosted and the cells transferred to a 15 ml Falcon™ tube using a pastette. Nine ml of cold (4°C) 199 growth media [199 Media base, (Eagle's Medium 10 X, Sigma-Aldrich) supplemented with 10% heat inactivated Foetal Bovine Serum (FBS)] (prepared as **Appendix 1.0**) were added to the tube.

The cells were pelleted at 280 x *g* for 5 min at 4°C (Jouan centrifuge model CR4.22). The supernatant was discarded in order to remove dimethyl sulfoxide (DMSO) cryoprotector and the cell pellet gently re-suspended in 1 ml of growth 199 media. The cells were then seeded in 30 ml 199 growth media in a 225 cm^2 polystyrene vented tissue culture flask (Corning Inc., NY. USA). Alternatively 2 ml of 199 growth media was added to the pelleted cells and the volume divided between two 150 cm^2 vented tissue culture flasks (Iwaki, Japan) containing 20 ml 199 growth media. In both cases the cells were subsequently grown in an atmosphere of 5% CO_2 at 37°C.

The flasks were checked every day under an inverted microscope until 80-100% confluence was reached. Once the desired confluence was observed, the media was decanted from the flask and the cell monolayer was washed with sterilized 1 X phosphate buffer saline (PBS) solution. For passaging the cells, versene solution containing 20% v/v of 0.25% w/v trypsin solution was added to the flasks in order to cover the monolayer surface. After two min incubation at 37°C, cells were detached from the flask by gentle agitation. An appropriate volume of fresh growth medium was added, mixed and the cell suspension divided between several new flasks depending on the size of flask. The re-seeded cells were incubated in an atmosphere of 5% CO_2 at 37°C.

2.3.1.2 SQPV infection of Foetal lamb skin cells (FLSc)

Generally, FLSc were infected with SQPV when the cell monolayer had reached a confluence of approximately 80%. Cells were washed twice with warmed (37°C)

sterile PBS prior to infection with virus. For a 225 cm^2 flasks, cells were infected with 4 ml of SQPV previously adapted in FLM cells culture (Red Squirrels SPPV 230, FLM 19, 10 Feb 1999), or 4 ml of maintenance 199 media [199 media base, (Eagle's Medium 10 X, Sigma-Aldrich) supplemented with 2% v/v FBS] (as illustrated in **Appendix 1.0**), as a mock control. Virus was allowed to adsorb for 1 hour at 37°C before the addition of 36 ml of maintenance 199 media.

In order to confirm the presence and integrity of infectious virus particles in the wild-type SQPV inoculum prepared for the time course infection experiment (**Chapter 5**), an aliquot was used to infect a culture of foetal lamb skin cells. One-hundred µl of SQPV inoculum (§**2.3.2**) was mixed with maintenance 199 media containing 0.5% v/v of 250µg/ml amphotericin B (Fungizone®, Sigma-Aldrich), 1% v/v 100U/ml polymixin B sulphate (Sigma-Aldrich) and 1% v/v of penicillin/streptomycin (100 units/ml and 0.1 g/l respectively), in a final volume of 2 ml. This virus/antibiotics mixture was filtered through a 0.45 µm sterilized Millipore filter prior to infection of the FLSc. The SQPV solution was divided equally in order to infect four of 25 cm^2 FLSc flasks. Additionally a mock infected flask was prepared as a negative control with the same amount of maintenance 199 media and antibiotics used for the SQPV infected flasks. In order to facilitate virus attachment to the cells, flasks were centrifuged at 1200 x *g* for 90 min at 26°C.

Infected and mock-infected cells were incubated in an atmosphere of 5% CO_2 at 37°C in maintenance 199 media from three to seven days and harvested when approximately 60% -80% cytopathic effect (CPE) was observed. Flasks containing the virus were then frozen at -70°C and thawed at 37°C three times in order to rupture the cell membranes and maximise the titre of infectious virions.

The virus/cell suspension was decanted into 50 ml conical centrifuge tubes and centrifuged at 2000 x *g* for 10 min at 4°C to pellet the cell debris. The supernatant was transferred into sterilized tubes and centrifuged at 119,046 x *g* for 45 min at 4°C (Kontron Type 45 fixed angle rotor, Optima L-90K centrifuge; Beckman Coulter, Brea, CA, USA). The resulting virus pellet was re-suspended in 1 ml of sterile PBS producing a concentrated SQPV preparation.

With the wild-type SQPV inoculum-infected cells, once CPE was observed, the flasks were again subjected to three freeze/thaw cycles and clarifying centrifugation, after which the resulting supernatant was used to infect fresh cells. This was to confirm that the observed CPE was caused by the virus itself and not by cytotoxicity due to cytolytic enzymes present in the wild-type virus inocula. The virus was passaged several times in this way to demonstrate the *in vitro* replication of the virus.

2.3.1.3 Viral titration using End Point Dilution assay ($TCID_{50\%}$)

A series of log10 dilutions (10^{-2} to 10^{-8}) of concentrated SQPV (Red Squirrels SPPV 230, FLM 19, 10 Feb 1999 as described in §**2.3.1.2**) were prepared in maintenance 199 media starting with a 10 µl volume. In order to check cell viability 15 µl of a cell suspension (FLSc) were taken and mixed with 15 µl of 0.1% (v/v) nigrosin in a 1.5 ml tube. Live cells were counted using an improved Neubauer counting chamber. Cells were counted in 25 fields and diluted in order to obtain the required concentration. One-hundred µl of cell suspension (FLSc) at a concentration of 2 x 10^5/ml were placed in each well of a 96-well format tissue-culture plate (Corning Inc., NY. USA). Immediately thereafter 25 µl of each virus dilution were inoculated into 12 replicates. Twelve cell-controls received 25 µl maintenance 199 media. The cells were incubated at 37°C and observed every day. Between 10 and 12 days the extent of the CPE was calculated. The Log $TCID_{50}$/ml was calculated using the Spearman/Karber method (Mahy & Kangro, 1996)

2.3.2 Preparation of virus inoculum from scabs

The following protocol was followed in order to produce crude wild-type virus inocula for the experimental infection studies. For specific details of these see individual chapters (**Chapters 4** and **5**).

Skin lesions and scabs were collected from red squirrels found with clinical signs of SQPV disease. Samples were previously analysed by negative staining transmission electron microscopy (EM) at the Veterinary Laboratories Agency (VLA), Weybridge, UK, to confirm poxvirus disease. Samples were stored at -70°C until preparation. Scabs were thawed and approximately 10 g were ground with sterile sand in 16 ml of sterile PBS, using a sterile pestle and mortar. When a smooth paste was formed another 16 ml of PBS were added to dilute the preparation. Two ml of

penicillin/streptomycin solution (100 units/ml and 100mg/ml respectively) was added to the final preparation (approximately 6% v/v) to prevent the growth of bacteria.

The ground scab was clarified by centrifuging at 2000 x *g* for 5 min. The supernatant was decanted into 1.8 ml tubes (Nunc ® CryoTube, Thermo Scientific, UK) and clarified again, this time at 1000 x *g* for 10 min. The supernatant was dispensed into one ml aliquots in fresh 1.8 ml tubes and stored at -70°C. A further clarifying centrifugation at 1000 x *g* for 10 min was performed prior to processing the virus for Real-time PCR analysis.

For the time course of infection study (**Chapter 5**) a new stock of SQPV inoculum was prepared. The same protocol was followed but with minor changes for scab selection by precisely collecting dry scab area only. In addition, the protocol for clarifying the supernatant was changed to include only one clarifying centrifugation step of 2000 x *g* for 5 min. The viability of the virus in the inoculum was assessed by growing in FLSc as outlined in §**2.3.1.2**.

2.4 Serology: SQPV ELISA

The direct enzyme-linked immunosorbent assay (ELISA) is a microtitre plate format assay that is used to detect, in this instance, IgG against SQPV contained in the sera and body cavity fluid of squirrels.

The tissue culture grown strain of squirrelpox virus (red squirrel 230), was used as the detecting antigen for the squirrelpox virus antibody ELISA. Duplicate wells of a 96 well plate (96 well flat bottomed, Greiner Bio-One, UK) were coated overnight at 4°C with 100 µl of 1/300 dilution of red squirrel antigen extracted from SQPV infected FLMc using the detergent IGEPAL® CA-630 (Sigma-Aldrich). Duplicate neighbouring wells were coated with a similar extract of mock infected FLM cells. The positive (SQPV) antigen and the negative antigen were diluted with ELISA coating buffer (bicarbonate/carbonate buffer pH 9.6, **Appendix 1.0**). Unbound antigen was removed by washing 4 times with ELISA wash buffer [1 X PBS with 0.05% v/v $Tween_{20}$ (PBST) (**Appendix 1.0**)]. Squirrel serum was diluted 1/50 with

ELISA diluent [1 X PBS / 0.05% v/v $Tween_{20}$ / 1% v/w bovine serum albumin (BSA)] (PBST 1% BSA, **Appendix 1.0**), and 100 µl of the diluted serum added to two sets of duplicate wells, two containing SQPV (positive) antigen and two containing control (negative) antigen. The serum was incubated at 37°C for 2 hours. After washing four times with ELISA wash buffer (PBST), 100 µl protein G conjugated to Horseradish peroxidise (HRP) (Sigma-Aldrich) diluted 1/3000 in ELISA dilution buffer (PBST 1% BSA), were added and plates incubated for a further 2 hours at 37°C. Protein G conjugated was used since has shown a high sensitivity to grey squirrel IgG (Sainsbury *et al*., 2000). After washing four times with PBST, 100 µl of the substrate TMB (Sure Blu™ TMB Microwell Peroxidase substrate, KPL, USA) were added to each well and incubated for no more than four to five min. The reaction was stopped adding 100 µl of 0.18 M H_2SO_4.

The optical density was read at 450 nm. The corrected optical density was calculated by subtracting the mean of the ODs from the two control wells from the mean OD_{450} from the wells containing the SQPV antigen (Sainsbury *et al*., 2000). A corrected OD_{450} value of >0.2 was considered as indicating the presence of specific antibody against SQPV.

2.5 Molecular methodology

2.5.1 DNA extraction

Isolation of total DNA from various sources such as squirrel tissues, cell culture grown virus, ethylenediamine tetra-acetic acid (EDTA) anticoagulated blood, cotton swabs, faeces and urine was performed using commercially available kits and reagents (Qiagen®) according to the manufacturer's instructions or adapted when necessary.

2.5.1.1 DNeasy® Blood and Tissue Kit

Total DNA extraction from solid tissue samples, tissue culture grown virus and the SQPV inocula was performed by using the DNeasy® Blood and Tissue Kit (Qiagen®).

Briefly, for solid tissues approximately 10-20 mg of tissue was placed in 2 ml microcentrifuge tubes containing homogenisation beads (Lysing Matrix D tubes, MP Biomedical, UK). Tissue disruption and lysis was achieved by adding 360µl of Qiagen ATL Buffer (DNeasy Blood & Tissue Kit, Qiagen®) to the samples and processing with a cell disruptor (Precellys® 24, PeqLab) set at 23 seconds for 6 cycles at 6200 rpm. The processing was paused for 2 min after the third cycle to avoid overheating of the samples. Proteins were digested by the addition of 40 µl of proteinase K (PK) solution (> 600 mAU/ml; approximately 20mg/ml; Qiagen®) and incubating for a 15 to 20 hours at 56°C until lysis was completed. To isolate nucleic acids, 800 µl of a solution containing 50% v/v of Qiagen AL buffer (DNeasy Blood and Tissue Kit; Qiagen®) in ethanol (99%) was added to the sample. The mixture was transferred to a DNeasy Mini Spin Column (Qiagen®) contained in a 2 ml collection tube. These columns contain a silica-gel membrane, which has a high and selective binding affinity for nucleic acids. Soluble proteins and divalent cations, which could inhibit subsequent PCR or other enzymatic reactions, do not bind to the silica filter and pass through the matrix. The column was centrifuged at 6000 x *g* for 1 minute, a process which was repeated twice in order to process the entire sample. The spin column was then washed using different buffers. First using buffer AW1 (Qiagen®) and after centrifugation at 6000 x *g* for 1 minute buffer, AW2 (Qiagen®) was added and the column centrifuged at 20,000 x *g* for 3 minutes in order remove salt and any residual contaminants. A further centrifugation at 20,000 x *g* for 1 minute was performed to remove all traces of fluid. The nucleic acids were then eluted from the silica filter with 100 µl Buffer AE (10 mM Tris-HCl; 0.5 mMEDTA; pH 9, Qiagen®).

Total DNA was obtained from both the SQPV inocula, prepared previously from scab materials or from the stock of tissue culture grown virus using the same protocol above, but with some minor changes. Thirty µl of the virus inoculum or media containing the infected cells, were added to 170 µl PBS to obtain a final volume of 200 µl. To perform cell lysis and disrupt the viral core, for the subsequent DNA purification, 20 µl Proteinase K solution (>600 mAU/ml; approximately 20mg/ml, Qiagen®) and 200 µl Buffer AL (DNeasy blood and Tissue Kit, Qiagen®) were added and mixed by vortexing. The solution was incubated at 56°C for 30 min.

Two hundred µl of ethanol (99% v/v) was added and mixed thoroughly by vortexing. DNA was then isolated according to the manufacturer's instructions (DNeasy blood and Tissue Kit, Qiagen®) as described in DNA extraction from scab material and tissues samples (see above).

2.5.1.2 QIAamp® DNA Mini kit

DNA was extracted from EDTA anticoagulated whole blood and dry cotton swabs following manufacturer's instructions as indicated in QIAamp® DNA Mini and Blood Mini handbook, 2007 (Qiagen®).

An internal positive control was included in the first nucleic acid extraction in order to confirm the accuracy of the methodology used and to assess the potential for PCR inhibitors being present in the samples. Specifically two different concentrations (2 µl diluted in sterile PBS at 1/10 and 1/40,) of SQPV virus inoculum (§**2.3.2**) were diluted in 400 µl of PBS and used to spike two sterilized cotton swabs. Nucleic acid extraction was then performed on the cotton swabs.

Cotton swabs were fully immersed in 1.8 ml microcentrifuge tubes containing a solution of 400 µl of PBS enriched with 400 µl of cell lysis buffer AL (Qiagen®) and 20 µl of PK. The tubes containing the cotton swabs were vortexed vigorously in order to loosen material from the cotton swabs and incubated at 56°C for 10 min to promote lysis. The same procedure was followed for DNA extraction from EDTA whole blood but 40 µl of PK were added to each sample instead. After a 10 min incubation period to degrade proteins, 400 µl of ethanol (99% v/v) were added to the homogenate solution. The mixture was applied to the QIAamp® spin column and centrifuged at 6000 x *g* for one minute. The column was washed using different buffers (Buffer AW1 and AW2, Qiagen®) in order remove salt and any residual contaminants. The DNA was eluted from the silica filter with 150 µl Buffer AE (10 mM Tris-HCl; 0.5 mMEDTA; pH 9, Qiagen®).

2.5.1.3 QIAamp® DNA stool Mini Kit

Because faecal samples contain several compounds that can degrade and consequently inhibit the PCR reaction, a different kit was used for DNA extraction from faecal samples. Nucleic acid extractions were performed using the QIAamp® DNA stool Mini Kit, according to the manufacturer's instructions.

Briefly, frozen faecal samples were quickly cut in order to have 200-220 mg to be processed using Buffer ASL, (Qiagen®) which removes the inhibitory substances. Microtubes containing the weighed samples were kept in ice to avoid thawing until the lysis buffer ASL was added. Samples were heated at 70°C for 5 min and were vortexed and then centrifuged at 20,000 x *g* for one minute in order to pellet the organic faecal material and cell debris present in the mixture. The supernatant was transferred to a clean microcentrifuge tube and PCR inhibitor substances and impurities were absorbed into an insoluble matrix by the addition of a tablet provided with the kit (InhibitEX Tablet, Qiagen®) by first vortexing the mixture for one minute and incubate at room temperature for an additional minute. Two-hundred µl of the liquid phase was mixed with same amount of buffer AL (Qiagen®) and 15 µl of PK and heated for further 10 min at 70°C to promote lysis and proteolysis. The mixture was cooled with the addition of 200 µl of ethanol (99% v/v) and then passed through the QIAamp kit column (Qiagen®). The column was then washed twice with buffers AW1 and AW2 (Qiagen®) as explained in §**2.5.1.1**, and the DNA eluted from the silica matrix with 200 µl Buffer AE (10 mM Tris-HCl; 0.5 mMEDTA; pH 9, Qiagen®).

2.5.1.4 QIAamp® Viral RNA Mini Kit

The protocol followed for DNA extraction from urine samples was adapted from that described in the QIAamp® Viral RNA Mini Handbook, 2007. Because the QIAamp® Viral RNA Mini Kit is not designed to separate viral RNA from cellular DNA, both nucleic acids will be present in the eluted sample. The protocol was therefore adapted to retain the DNA and steps recommended for RNA extraction were omitted. Additionally, no concentration of samples was performed. To confirm that the protocol used was suitable for DNA extraction from urine samples that may contain several PCR inhibitors, an aliquot of squirrel urine was spiked with two different amounts of SQPV (see dilution used for cotton swabs in §**2.5.1.2**) as internal positive control for nucleic acid extraction.

One-hundred and forty µl of urine were added to 560 µl of prepared AVL buffer containing an AVE buffer–carrier RNA (Qiagen®) mixture in a 1.8 ml microtube. The mixture was incubated for 10 min at room temperature (20°C) and then 560 µl of

ethanol (99%) were added. All of the mixture was transferred to the QIAamp Mini column (Qiagen®) in two steps (as in §**2.5.1.1**) with the matrix being washed twice with Buffer AW1 and AW2 (Qiagen®). An additional, recommended centrifugation step at 20,000 x *g* for 1 min after the wash with buffer AW2 was performed. The DNA was eluted in two separate steps each with 60 µl of buffer AVE to give a final volume of 120 µl.

2.5.2 Spectrophotometric quantification of DNA

Extracted DNA was quantified by spectrophotometry at 260/280 nm using a NanoDrop® (ND-1000) spectrophotometer (Labtech International Ltd., UK). Sterile water was used as a blank.

2.5.3 Polymerase chain reaction

The polymerase chain reaction (PCR) was used to amplify specific sequences of SQPV DNA for cloning proposes (**Chapter 6**) and for screening DNA samples for detection of squirrelpox viral DNA (**Chapter 3** and **4)**.

For cloning specific genes to determine variability between different isolates of virus (**Chapter 6**), oligonucleotide primers were designed using DNASTAR Lasergene Version 8.0 package software and the SQPV 1296/99 sequence deposited in the GenBank database under accession numbers DQ377804 or DQ377805 (**Table 2.1**). The primers were designed to cover the entire coding region and flanking regions of the three genes of interest (O8R, C1R and A6L) as outlined in **Table 2.1**. The primers were synthesized and purified at MGW (Eurofins MGW Operons, UK) and the lyophilized pellet re-suspended in distilled molecular grade DNase/RNase free water in order to obtain a final stock concentration of 100 µM.

Table 2.1 Primer sets used for cloning purposes.

Primers name	Primers Sequence	Target genes	Amplicon size (bp)	ORF length (bp)
→ sqpvO8RF	5'-CTGTCGTTGTTGATCTTCCCTCA-3'	O8R	678	582
← sqpvO8RR	5'-TCATCATGGCCATTTATTACACAA-3'			
→ sqpvC1RF	5'-AGTGAGGTGAGAGGCTACTACTGC-3'	C1R	669	561
← sqpvC1RR	5'-CGGCATATAGGTAAGCGGGATTA-3'			
→ sqpvA6LF	5'-GGAGTGACATCCCGAGGTGAAA-3'	A6L	1252	1158
← sqpvA6LR	5'-CGCGCGCTAGGAAACCCAAC-3'			

Primers sequences and their relative amplicon products sizes for PCR analysis of each open reading frame (ORF) of interest.

Using the method of McInnes *et al*., (2006), the A11L gene of SQPV was amplified using the primers "SQ026F" 5'-ATGTCAGTCACGATAAGATT-3' and "SQ026R" 5'-TCATGTCAGTCGGGTGATGA-3' that were designed to amplify a 258 bp fragment of the SQPV genome. This was used as a rapid screening method to ensure that SQPV DNA was present in the sample being tested.

The standard PCR was performed in a total volume of 25 µl using 1 µl of extracted DNA template. The DNA was incubated with one unit of Taq DNA Polymerase (Roche), 1µM of each desired PCR primers, 200 µM of deoxynucleotides (dNTPs) supplied by Roche, and the 1X PCR reaction buffer (1·5mM $MgCl_2$, 10mM Tris-HCl, pH 8·3 and 50mM KCl) supplied by Roche.

All PCR reactions were prepared in a 0.2 ml thin-walled PCR tubes (DNase/RNase free (Elkay Laboratory products, Hampshire, UK) following the protocol indicated in **Table 2.2**.

Table 2.2 PCR protocol.

Reagents	Volume in µl
Forward Primer (10µM)	2.5
Reverse Primer (10µM)	2.5
10X dNTP mixture (5 µM)	1
10X PCR reaction buffer	2.5
Taq DNA polymerase (5U/µl)	0.5
Distilled DNase/RNase free water	15
DNA template	1

All PCR cycling was performed on an MBS Satellite 0.2G Thermocycler (Thermo Electron Corporation, Waltham, MA, USA) as follows: DNA was denatured by incubating at 95°C for 1 min and amplified using 30 cycles of 94°C for 1 min, 55°C or 60°C for 30 sec (primer annealing) and 72°C for 1 min, followed by a final incubation at 72°C for 5 min. Specific cycling conditions for each of the PCR assays are given in **Tables 2.3** and **2.4**.

Table 2.3 PCR protocol condition reaction forA11L, O8R and C1R genes.

	STAGE 1		STAGE 2		STAGE 3
		1st step	2nd step	3rd step	
Temperature (°C)	95	94	55	72	72
Cycles	1	30	30	30	1
Time(h:m:s)	00:05:00	00:01:00	00:00:30	00:01:00	00:05:00

Table 2.4 PCR protocol condition reaction A6L gene

	STAGE 1		STAGE 2		STAGE 3
		1st step	2nd step	3rd step	
Temperature (°C)	95	94	60	72	72
Cycles	1	30	30	30	1
Time(h:m:s)	00:05:00	00:01:00	00:00:30	00:01:00	00:05:00

The amplification products were electrophoresed in a 1.5% w/v agarose gel and visualized under UV illumination (§**2.5.4**).

2.5.4 Visualization of DNA from agarose gel by electrophoresis

DNA fragments were verified by agarose gel electrophoresis and visualized by ultra violet light (UV) transillumination. The concentration of agarose in electrophoresis 1 x TBE buffer (0.9M Tris-borate, 2mM EDTA, pH 8, see **Appendix 1.0**) was selected in accordance with the fragment to be resolved, but generally 1.5% (w/v dissolved in electrophoresis buffer) was used. To allow visualization of nucleic acids, GelRed™ (Biotium, Cambridge, UK) was added to the molten agarose at a concentration of 0.01% (v/v) (5 µl in 50 ml of buffer TBE plus agar). DNA samples were mixed with 6 X loading buffer [30% glycerol v/v, 0.25% bromophenol blue, 0.25% xylene cyanol FF, see **Appendix 1.0**] to a final concentration of 1 X before loading into agarose gel. Samples were electrophoresed alongside a known standard (1kb DNA ladder, Invitrogen; 1 kb plus DNA ladder, Invitrogen; 1 kb DNA ladder, Promega, 100bp DNA ladder, Invitrogen) in order to verify the size length of the DNA in the

test sample. Gel electrophoresis was performed in 1 x TBE buffer in electrophoresis unit at 80 volts for ca. 45 min. DNA fragments were visualized by ultraviolet light (UV) transillumination and images were photographed or digitalized.

2.5.5 PCR product purification

Where downstream analysis was required (**Chapter 6**), PCR products were purified to remove excess primers, nucleotides, and salt using a commercial kit (QIAquick PCR purification kit [Qiagen®]) following the manufacturer's instructions.

Before starting, an optimal pH indicator (pH indicator I, Qiagen®) was added to the binding buffer PB to enables the checking of the optimal pH required for DNA adsorption (≤7.5) in the reaction. Briefly, 125 µl of buffer PB (Qiagen®), were added to 25µl of the PCR sample and mixed. The buffer PB allows the efficient binding of cDNA and removal of oligonucleotides primers still present in the PCR products. To bind the DNA the sample was transferred in a QIAquick spin column and centrifuged for 1 min. at 17,900 x *g*. The column was washed with 750 µl of buffer PE (Qiagen®) and centrifuged for one min at 17,900 x *g*. An additional centrifugation step was performed for another minute. The spin column was transferred to a 1.8 ml microcentrifuge tube and the DNA was eluted with 50 µl of buffer EB (10mM tris-Cl, Ph 8.5, Qiagen®) and centrifuged as before.

2.5.6 Isolation of DNA from agarose gels

The DNA was extracted from agarose gels using the commercial kit QIAquick® gel extraction kit (Qiagen®), following the manufacturer's instructions.

The gel was illuminated using a UV light (302 nm) in order to make DNA visible and the required DNA band was excised from the gel using a scalpel blade. The gel piece was transferred to a 1.8 ml tube and weighed. Three gel volumes of buffer QG was added to the tube and incubated at 50°C for 15 min until agarose gel was completely dissolved. The solution was then applied to the QIAquick column and centrifuged at 17,900 x *g* for 1 min. The column was washed using 750 µl of buffer PE by centrifuging as before and then placed in a clean collection tube and centrifuged for a further minute to ensure the remove of ethanol traces. DNA was eluted by adding 30 µl of buffer EB (10mM Tris-Cl, pH 8.5, Qiagen®) to the column

membrane and incubated for one minute before centrifugation as before in another clean 1.8 ml micro-centrifuge tube.

2.5.7 TaqMan® Real-time PCR (qPCR)

Total DNA extracted from each tissue was quantified by spectrophotometry (§**2.5.2**). The concentration of each sample was adjusted, when necessary, with RNase/DNase-free water, to a final concentration of 40 ng/µl. The final concentration used in each qPCR reaction was 200 ng per well.

A TaqMan® Real-time PCR (qPCR) assay was used to detect SQPV DNA. The qPCR assay was based on the amplification of part of the SQPV H2L or I1L genes. These genes were identified (McInnes *et al*., 2006) from SQPV isolated during an epidemic of pox-like disease in northeast England in 1999 (Thomas *et al*., 2003). The SQPV H2L gene encodes an RNA polymerase subunit (vaccinia virus: VACV-Cop E4L homolog) and the I1L which encodes a possible orthologue of a *Molluscum contagiosum virus* (MOCV) gene with a predicted function of being an immunoglobulin domain.

Table 2.5 TaqMan® Real-time PCR primers and Probes (5'→3').

Gene	Forward primer	Probe	Reverse primer
H2L	AGTACACGGACCTCTGCGACAT	JOE-ACATCCTGCGCTACGGCCTCT-TAMRA	CCCTTTCGCAGGCACTTG
I1L	TCCTGCAGTCATCCATCGAA	6FAM-CTCCGATCCCCGTCGCAACCT-TAMRA	TCGCTGATGTTGTAGATGAAGTTG

The SQPV H2L probe has the fluorescent reporter dye JOE™ located at the 5' end of the probe and the quencher Tetramethylrhodamine (TAMRA) at the 3' end.

SQPV I1L probe has the reporter dye 6-carboxy-fluorescein (6-FAM) located at the 5' end of the probe and the quencher Tetramethylrhodamine (TAMRA) at the 3' end.

The TaqMan® Real-time PCR was carried out as follows: reaction conditions were established for the Applied Biosystem 7000 Sequence Detection System and were performed in optical quality 96 semi-skirted well ABgene® PCR plates (Thermo Scientific, UK) in a total reaction volume of 25 µl; 12.5 µl of 2 x TaqMan® Universal PCR Master Mix (Applied Biosystem) was used per reaction and specific gene primers and probes (**Table 2.5**) were added to the Master Mix. For H2L, primers and probes were added to achieve a final concentration of 0.9 µM (forward and reverse primers) and 0.25 µM respectively. For I1L, primers and probe were added to achieve a final concentration of 0.3 µM (forward primer), 0.9 µM (reverse primer) and 0.2 µM for the I1L probe; 5 µl of DNA template (40 ng/µl) were added.

All samples were tested in quadruplicate with negative and positive PCR controls; for the positive controls viral genomic DNA prepared from scab material or a SQPV cosmid vector containing the appropriate region of the SQPV genome were used; distilled water was used as a no template (negative) control.

Reaction conditions were as follows; 2 min incubation at 50°C, 10 min at 95°C for denaturation of the DNA and forty-five cycles each consisting of 95°C for 15 sec and 60°C for 1 min for denaturation and annealing/extension. After the final cycle, the reaction was cooled to 30°C.

Data evaluation was carried out using the software installed on the PCR machine (ABI PRISM® 7000 System SDS software core Application, Version 1.2.3., Applied

Biosystem) and Microsoft® Excel program. The threshold crossing point cycle (Cp) value was determined for all qPCR reactions and the mean value of the replicate results was recorded. The crossing point (Cp) value is inversely proportional to the log of the amount of target DNA initially present and represents the cycle number at which the amplification plot, representing the fluorescence emission of the reporter dye, passes a fixed threshold. The threshold was automatically set at 0.2 above the mean baseline emission. In most cases the threshold was manually adjusted within the logarithmic curve, above background level and below the plateau phase to ensure that analysis was carried out using the exponential stage of amplification.

2.5.7.1 Construction of qPCR Standard Curve

To enable absolute quantification of viral DNA qPCR assay was used to amplify and detect the H2L or I1L regions of SQPV genome cloned into a cosmid vector (SQPV cosmid #86). Ten-fold serial dilutions of SQPV cosmid #86, ranging from 3×10^7 to 3 genome equivalents were used to determine the dynamic range of quantification. The serial dilutions of this cosmid were also used to establish the sensitivity of qPCR.

The mass of a single SQPV cosmid #86 molecule was calculated as follows; the cosmid #86 size (39650 bp) was multiplied by the mass of dsDNA molecule (1.09×10^{-21} g/bp) to give the mass of the single SQPV cosmid #86 of 4.35×10^{-17} g. This value was then multiplied by the copy number of the gene of interest (3×10^7 to 3 genome equivalents) in order to obtain the mass of SQPV cosmid #86 DNA needed for each individual dilution (**Table 2.6**).

Table 2.6 Mass equivalent of SQPV cosmid #86 needed for each copy number of target gene.

Copy number of gene	Mass of single SQPV cosmid #86	Mass of SQPV cosmid#86 needed (g)
30000000	4.35×10^{-17}	1.3×10^{-9}
3000000	4.35×10^{-17}	1.3×10^{-10}
300000	4.35×10^{-17}	1.3×10^{-11}
30000	4.35×10^{-17}	1.3×10^{-12}
3000	4.35×10^{-17}	1.3×10^{-13}
300	4.35×10^{-17}	1.3×10^{-14}
30	4.35×10^{-17}	1.3×10^{-15}
3	4.35×10^{-17}	1.3×10^{-16}

Once the mass of SQPV cosmid #86 was calculated for each dilution, this was divided by the volume (µl) required for each qPCR reaction. The concentration of a stock of SQPV cosmid #86 was measured using spectrophotometry. It was consequently diluted to give the required concentration for the highest copy number (3×10^7); that concentration was serially diluted through eight orders of magnitude.

Table 2.7 Calculated volumes of SQPV cosmid #86 and diluents for the eight orders of magnitude.

Dilution	Source of cosmid	Initial concentration (ng/µl)	Volume of cosmid (µl)	Volume of water (µl)	Final volume (µl)	Final cosmid concentration (ng/µl)	Copy of gene
1/100	Uncut Cosmid#86	530	1	99	100	5.3	
1	1/100	5.3	12.26	37.74	50	1.3	$3x\ 10^{7}$
2	0.10	1.3	2	18	20	$1.3x\ 10^{-1}$	$3x\ 10^{6}$
3	0.20	$1.3x\ 10^{-1}$	2	18	20	$1.3x\ 10^{-2}$	$3x\ 10^{5}$
4	0.30	$1.3x\ 10^{-2}$	2	18	20	$1.3x\ 10^{-3}$	$3x\ 10^{4}$
5	0.40	$1.3x\ 10^{-3}$	2	18	20	$1.3x\ 10^{-4}$	$3x\ 10^{3}$
6	0.50	$1.3x\ 10^{-4}$	2	18	20	$1.3x\ 10^{-5}$	$3x\ 10^{2}$
7	0.60	$1.3x\ 10^{-5}$	2	18	20	$1.3x\ 10^{-6}$	30
8	0.70	$1.3x\ 10^{-6}$	2	18	20	$1.3x\ 10^{-7}$	3

In order to keep the total DNA concentration approximately the same in each reaction, 10 µl of each dilution (**Table 2.7** column six) were added to 10 µl bovine genomic DNA (200 ng/µl). The qPCR protocol was followed as indicated in §**2.5.7** and 2 µl of that final solution was added to each well.

2.5.8 Molecular cloning

2.5.8.1 Cloning vector

The pGEM®-T easy Vector System (Promega) was used for cloning proposes. PCR products amplified from the SQPV genome (§**2.5.3**) were purified (§**2.5.5**) and their concentrations calculated using a spectrophotometer (§**2.5.2**). The DNA fragments obtained (insert) were then cloned into the linearised pGEM®-T easy Vector System (Promega) (**Figure 2.1**) as per the manufacturer's instructions.

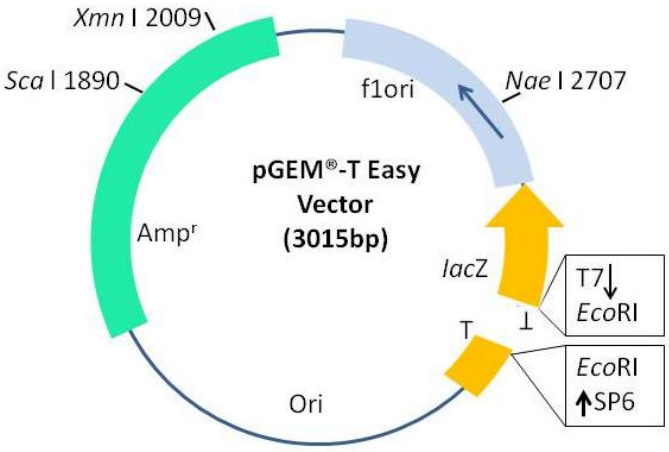


Figure 2.1 Circular map of pGEM-T Easy Vector.

The vector is 3015 bp in length and is prepared by cutting with *Eco*RV enzyme and the addition of 3' terminal thymidine to both ends (two complementary T overhangs at the insertion site within the *Iac*Z gene, coloured in yellow). Positions of T7 and SP6 RNA promoters are indicated in the right hand squares flanking the multiple cloning regions consisting of restriction sites conventionally arranged. In this figure only *Eco*RI is represented.

The pGEM®-T easy Vector contains T7 and SP6 RNA polymerase promoters and an α-peptide coding region of the enzyme ß-galactosidase. The multiple cloning region is situated within the plasmid *LacZ* gene and insertional inactivation of the α-peptide allows recombinant clones to be selected by blue/white colony colour selection on indicator plates. In the pGEM®-T easy Vector the single 3'-T overhangs at the insertion site improve efficiency of ligation of a PCR product. The vector contains an ampicillin resistance gene (**Figure 2.1** light green colour) permitting selection of transformed colonies by the addition of ampicillin to the agar plates.

2.5.8.2 Ligation

Ligation was performed according to the manufacturer's instructions.

Briefly, the insert DNA was diluted in order to have a molar ratio of PCR product: vector of 3:1 following the equation:

$$\frac{\text{Ng of vector} \times \text{Kb size of insert}}{\text{Kb size of vector}} \times \text{insert: vector molar ratio} = \text{Ng of interest}$$

Ligation reactions were performed in 0.5 ml tubes having low DNA-binding capacity and the mixture was prepared as shown in **Table 2.8**. The insert and the plasmid DNA were incubated with the T4 DNA ligase (Promega) enzyme to form a covalent bond between the ends.

Table 2.8 Table illustrating the set up of ligation reaction using pGEM®-T Easy Vector.

	A6L	O8R	C1R
2X Rapid Ligation Buffer, T4 DNA ligase	5µl	5µl	5µl
pGEM-T® Easy vector (50ng)	1 µl	1 µl	1 µl
PCR product	x µl*	x µl*	x µl*
Control insert DNA	-	-	-
T4 DNA ligase (3/Weissunit/ µl)	1 µl	1 µl	1 µl
Deionised water to final volume of 10 µl	x µl**	x µl**	x µl**

*Molar ratio of each PCR product: vector were optimized at 3:1
** Vary according to the µl used for the PCR product

Reactions were incubated overnight at 4°C to maximise the efficiency of the ligation reaction.

2.5.8.3 Transformation of competent JM109 E. coli cells

The *Escherichia coli* strain JM109 was transformed by the recombinant DNA (insert and vector). Two µl of each ligation reaction were mixed and incubated on ice for 20 min with 50 µl or 65 µl of competent cells. The mixture was heat-shocked at 42°C

for 45-50 sec and returned to ice for a further 2 min. Six hundred and fifty µl of Luria-Bertani broth (LB), as shown in **Appendix 1.0**, at 24°C were added to the tubes containing cells and incubated for 1.5 hours at 37°C in a shaking incubator. One hundred µl of each transformation culture was spread over the surface of LB agar plate supplemented with 50 µg/ml ampicillin, 100 µl of 100 mM (0.1M) IPTG and 20 µl of 50 mg/ml X-Gal (Appendix 1.0 §**2.1.3**). The plate was inverted and incubated overnight at 37°C.

2.5.8.4 Selection, amplification and harvesting of clones

With a sterile wooden stick (e.g. toothpick) a single white colony from the transformation reaction was inoculated into 10 ml of LB medium containing 50 µg/ml of ampicillin. At least four individual colonies were taken from each transformation plate. The inoculated samples were incubated at 37°C overnight (no more than 15 hours) with vigorous shaking.

Each of the overnight bacterial cultures were aliquoted into two of 1.8 ml microtubes per sample and bacterial cells harvested by centrifugation at 6800 x *g* for 5 min at 22°C.

2.5.8.5 Plasmid DNA purification: miniprep

This protocol was used to extract dsDNA plasmid for restriction enzyme digestion and DNA sequencing. Normally no more than 12 plasmid preparations were performed at the same time.

Up to 20 µg of plasmid DNA could be obtained from 3.6 ml overnight culture of selected clones using the QIAprep spin Miniprep® kit (Qiagen®). The procedure was carried out in accordance with the manufacturer's instructions using the buffers supplied with the kit.

Briefly, the bacterial pellet was re-suspended in 250 µl of Buffer P1 (Qiagen®) and then 250 µl of buffer P2 was added and mixed by inverting the tube 5-6 times. The lysis reaction was not allowed to proceed for more than 5 min. Bacterial debris and protein were precipitated by adding 350 µl of buffer N3 (Qiagen®), the mixture was mixed gently by inverting tubes 4-5 times and the precipitate pelleted by centrifugation at 17,900 x *g* for 10 min. The supernatant was applied to the spin column (QIAprep spin column, Qiagen®) by pipetting and centrifuging for 1 min.

The column was washed first with 500µl of buffer PB followed by centrifugation for 1 min and then with 750µl of buffer PE (Qiagen®) followed by centrifugation twice (each for 1 min) to remove all the residual ethanol present in the column. DNA was eluted from the column by incubating for 1 min with 50 µl buffer EB (10mM Tris-Cl, pH 8.5, Qiagen®) followed by centrifugation for 1 min. The eluted DNA was stored at -20°C.

2.5.8.6 Restriction endonuclease digests

Since in the pGEM-T easy vector the inserted PCR product is flanked by *Eco*RI sites (**Figure 2.1**) this enzyme was used to restrict the miniprep DNA to identify clones that contained the DNA fragment of the anticipated size.

Restriction digest with *Eco*RI was performed with 1 U of enzyme per 0.2-1.0 µg DNA in a total volume of 20 µl reaction for 2 hours at 37°C. The specific buffer given for the enzyme was supplied at 10X concentration and therefore diluted to 1 X in the reaction mixture. The digested DNA was electrophoresed through a 1.5% agarose gel and the size of the cloned insert assessed by comparison with known size standards (§**2.5.4**).

2.5.8.7 Generation of DNA Sequence and its analysis

Plasmid DNA samples (template concentration 100 ng/µl) containing inserts of the correct size were submitted to the Genomics Service Company at Cogenics Technologies, Essex, UK (isolate R597/07) and all others were submitted to the Eurofins MGW GmbH Sequence Service, Ebersberg, Germany. Sequences were requested from both directions using sequencing primers corresponding to the T7 and SP6 regions of the plasmid. Three clones for each gene from each different virus isolate were sequenced.

Six separate sequences (three forward and three reverse) were generated for each gene from each virus isolate. The consensus sequence of individual genes was produced by assembling the six individual sequences using the Seqman program in the Lasergene software suite (DNASTAR, Lasergene Version 8.0) and editing with the help of the Chromas Lite Version 2.01 graphical software package (Technelysium Pty Ltd). The sequences were edited to remove both vector and primer sequences. Once the consensus sequence for the cloned fragment (gene) was

obtained it was used to interrogate the GenBank nucleotide sequence database using the BLAST algorithm (Altshul *et al*., 1990) to ensure that the appropriate target gene had been amplified, cloned and sequenced.

The nucleotide sequences were translated to produce predicted amino acid sequences using Seqbuilder software (DNASTAR, Lasergene Version 8.0). The nucleotide and predicted amino acid sequences for each gene from eight virus isolates were then assembled into a multiple sequence aligment using the MegAlign programme (using the ClustalW2 algorithm) of the DNASTAR sequence analysis package, Version 8.0.

2.6 Experimental infection of grey squirrels

2.6.1 Animals

A total of 60 adult and sub-adult male and female wild grey squirrels (*Sciurus carolinensis*) were used for the experimental infections under licence from the Home Office and approved by the Moredun Research Institute local ethics committee. The animals were humanely trapped during the south Scotland grey squirrel control program near Hawick, in the Scottish Borders. All the procedures after the animals' arrival, including post mortem examination and disposal of carcasses were performed in a microbiologically secure (Biocontainment level 3) animal room at the Moredun Research Institute (MRI, Scotland, UK).

2.6.2 Housing conditions

Alphabetic letters were used to uniquely identify each squirrel. The squirrels were individually housed in a steel rack cage (1 m x 1 m^2 floor area) in two tiers and supported in movable frames. Each cage was furnished with a cubical nest box with a removable lid. Each nest was insulated on the inside by wooden panels. The environment of the cages was enriched with a variety of materials such as cardboard tunnels, wood stick tunnels, strips of paper for the nest, wooden baskets and pieces of wood for climbing (**Figure 2.2**).

Figure 2.2 Squirrel cage and nesting environment.

Top left picture represents an example of individual squirrel cage enriched with materials such strips of paper for nesting, hanging wooden basket and cubical nest box. The top right picture shows a squirrel in a wood tunnel within the cage. The bottom left picture shows multiple cubical nest boxes with removable lid and the wood panel inside. The bottom right picture shows a squirrel inside a cubical nest box within the cage.

Sawdust was placed in a removable panel under each cage to aid removal of faeces and urine. The room was maintained at the constant temperature of 20 ± 2°C and humidity of 38 ± 3%.

On the day of capture all squirrels were treated for ecto- and endoparasites using 2.5 ml of Oramec Drench® (Ivermectin 0.08% w/v; Merial Animal Health Ltd) added to 500 ml of drinking water. The Ivermectin was added to drinking water for two consecutive weeks. Food (mixed nuts, sunflower seeds, whole corn, and fruit) and water were provided *ad libitum* for the duration of the experiment. All squirrels were allowed to adjust to captivity for one week before the start of the procedures.

2.6.3 Handling

All handling procedures, for example, withdrawal of blood, infection with SQPV or mock challenge and euthanasia were performed under general anaesthesia. All animals were physically restrained with inhaled isoflurane ($CF_3CHCL\text{-}O\text{-}CF_2H$) 5% (IsoFlo®, Abbott Animal Health, UK) in oxygen (4 l/min) administered within a gas box (**Figure 2.3**). Anaesthesia was maintained by intramuscular injection in the hind limb of 0.2 ml of 100 mg/ml of ketamine hydrochloride (Ketaset®, Fort Dodge Animal Health).

Figure 2.3 Gas box used to anesthetise squirrels.

Picture showing the gas anaesthetic equipment used to administer isoflurane to squirrels. Each squirrel was transported from its cage to inside the gas box using the cubic nest box. The gas box was filled with isoflurane. The squirrels were removed from the cubic nest when lightly anaesthetised and ketamine hydrochloride given by intramuscular injection.

2.6.4 Clinical examination

It was not technically possible to examine or weigh the squirrels on their own without anaesthesia. Therefore body mass measurements were taken by weighing squirrels in their nest boxes every three days. Animals were also weighed directly when possible under anaesthesia (**Figure 2.4**).

Figure 2.4 Measurement of squirrel body mass.

The body mass of grey squirrels was estimated in two ways. Every three days the squirrel was weighed in its nest box, (left-hand picture). The squirrels' weight was calculated by subtracting the weight of the nest box (determined before the start of the experiment) from the weight indicated by the digital balance. The right-hand picture illustrates the fact that under anaesthesia it was possible to weigh the squirrel directly.

During the course of experiments, animals were examined daily while free in the cage for clinical signs of disease. Weight was recorded at least every three days throughout the experiment. A daily clinical scoring system, modified from that used to assess the impact of squirrelpox disease on red squirrels (Tompkins *et al*., 2002), was used to assess health of the grey squirrels following the infections. Lack of faeces and/or urine was added as a further clinical parameter for evaluation (**Table 2.9**). A total clinical score of eight points on three consecutive days was chosen as the humane end-point at which an individual animal would be removed from the experiment. Daily clinical scores were recorded for each animal on a score sheet (**Figure 2.5**)

Table 2.9 Clinical scoring system used to assess the impact of SQPV on experimentally infected grey squirrels.

Assessment category	Clinical observation	Clinical score
Size of poxvirus lesion	< 1 mm diameter	1
	≥ 1 mm and < 3 mm diameter	2
	≥ 3 mm diameter	3
Poxvirus lesion at distal sites	One other location	1
	Two or more other locations	2
Depression	Animal "sick" looking (lethargic, poor condition...)	1
	Lack of faeces/urine	1
	Appetite loss (>50% reduction in food consumption)	2
	Complete loss of appetite	3
Weight loss	≥ 10% and <20% weight loss	2
	≥ 20% and < 30% weight loss	4
	≥ 30% weight loss	8

Date:

Squirrel ID	Pox lesion	Distal lesion	Appetite/ depression	Faeces/ urine	Weight	Weight loss score	TOTAL SCORE

Notes:

Figure 2.5 Daily sheet used to score squirrels during the experiments.

2.6.5 Virus challenge

For the experiment described in **Chapter 4**, squirrels were challenged with SQPV using three different routes of infection: namely intranasal (I/N), subcutaneous (S/C) and epidermal scarification (SCA) of the hind leg, whereas animals used for the time course experiment, as described in **Chapter 5**, were challenged only via scarification. For the time course experiment some animals were also mock scarified on the opposite hind leg with 100 µl of mock preparation. For the mock infection the inoculum was prepared using the same SQPV wild-type inoculum protocol (§**2.3.2**), but with PBS added to the antibiotics solution instead of the SQPV inoculum.

For all routes of infection animals were initially anesthetized and then challenged with 100 µl of SQPV inoculum or 100 µl of mock inoculum. The viral concentrations/ml were estimated as approximately 1.67 x 10^8 viruses/ml for the first challenge; a minimum of 1.5 x 10^6 $TCID_{50}$/ml for the second challenge as described in **Chapter 4;** and 5 x 10^{10} viruses/ml for the challenge in the experiment described in **Chapter 5**.

For the scarification challenge SQPV or mock inocula were dripped topically onto a region of shaved and scratched skin on the leg using the tip of a 16 gauge needle (**Figure 2.6**). The operation was completed by a further gentle scratching of the area in order to ensure that the liquid inoculum was absorbed into the skin.

SQPV intranasal inoculation was performed using a rubber catheter attached to the end of a 1 ml syringe barrel containing the inoculum. The other end of the catheter was inserted into the squirrel's nostril. A gentle pressure was applied to the syringe plunger to eject the inocula and the animals were kept in a dorsal recumbent position for approximately 1 min.

For the subcutaneous route, animals were challenged by subcutaneous injection of 100 µl of inoculum into the interscapular area using a 1 ml syringe.

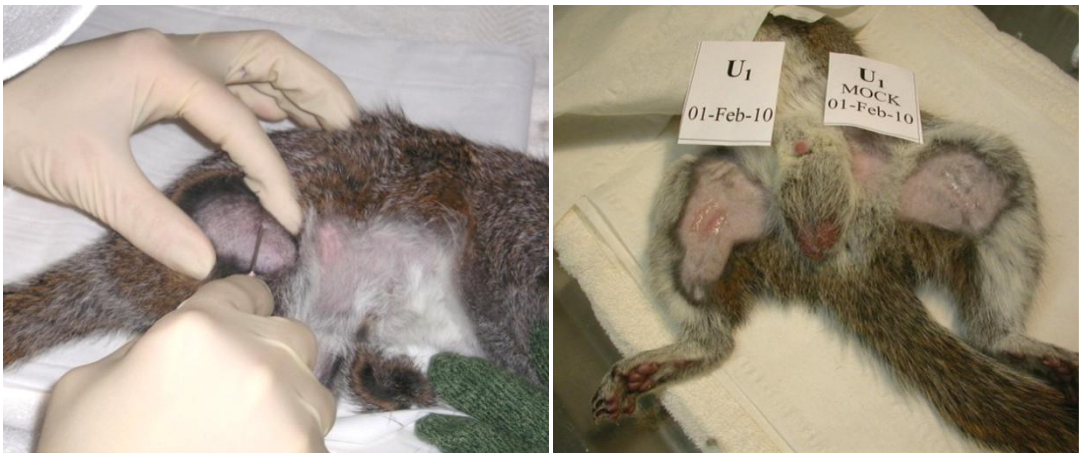


Figure 2.6 Scarification technique.

Two examples of scarification technique performed for the experimental infections. Picture on the left-hand shows the scarification procedure utilized to challenge animals for the routes of infection experiment and for the time course (long term) experiment. For the time course experiment (short term), squirrels were scarified on the medial aspect of the thigh as shown in the right-hand picture.

2.6.6 Sample collection

2.6.6.1 Blood sampling and preparation of sera.

Prior to SQPV challenge, between 200 µl and 400 µl of blood was collected from the femoral vein of each squirrel using a 25 gauge hypodermic needle (**Figure 2.7**). This was used to determine their pre-experiment sero-status for SQPV. Thereafter, during the experiments, blood was collected on a two-weekly basis until the end of the experiment (for the route of infection experiment, **Chapter 4**) or in the case of the time course experiment (**Chapter 5**), blood was collected during the first three weeks when individual squirrels were euthanized. The maximum amount of blood collected from each animal at each time point was 500 µl.

For the time course experiment (**Chapter 5**) an aliquot of blood was placed in a paediatric EDTA tube (Teklab, Medical Laboratories Ltd, UK) (**Figure 2.7**) and the remaining blood was allowed to clot in microcentrifuge polypropylene tubes at room temperature (20-22°C) for approximately four hours and then left at 4°C over night. Serum was separated from the clot by centrifugation at 2000 x *g* for 5 min. at 4°C. The resulting sera were screened for the presence of antibodies to SQPV by direct ELISA (**Appendix 1.0**).

As a terminal procedure, 5 or 6 ml of blood was collected by cardiac puncture using a 21 gauge hypodermic needle. This blood was divided into two aliquots; one was allowed to clot for serology and the other aliquot was collected in EDTA tubes for nucleic acid extraction (**Figure. 2.7**).

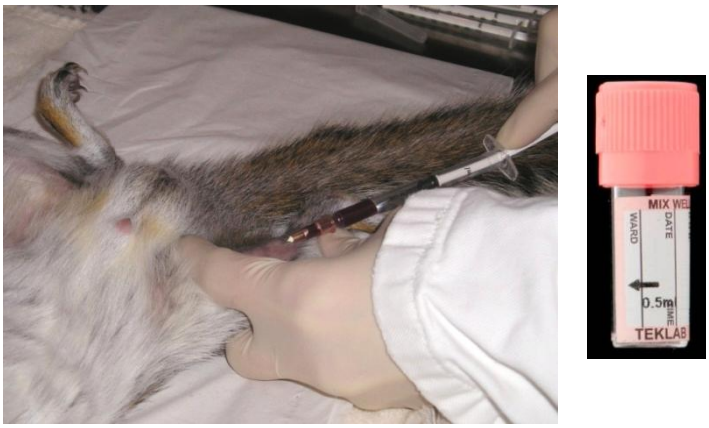


Figure 2.7 Blood sampling.

Blood sampling from the femoral vein (left) and paediatric haematology tube containing EDTA used to collect blood sample.

2.6.6.2 Collection of tissues and other organ samples

Tissue samples were collected immediately following euthanasia. Each tissue sample was collected in duplicate using sterile 1.8 ml screw cap (Nunc Cryo tube™). One of the duplicate samples was used for subsequent molecular analysis whilst the other was fixed in 10% v/v formal saline solution (**Appendix 1.0**) for histopathological examination. Sterilized surgical instruments were changed between each tissue sample in order to avoid cross-contamination.

Urine samples were usually collected by cystocentesis during the post mortem examination. On several occasions, as a result of incontinence due to the urinary sphincter relaxation, urine was collected directly from the urethral opening. Faecal samples were obtained from the rectum. In the lack of formed faecal pellets in the distal large intestine, faecal material was collected from the caecum.

2.6.6.3 Swabs

Oral and ocular secretions were obtained under anaesthesia, prior to euthanasia using sterile cotton-tipped swabs. Oral swabs were collected by inserting the swab into the mouth and gently rubbing inside the oral cavity, including the lateral, dorsal and ventral aspects of the oral cavity. For ocular swabs, a few drops of sterilized PBS were dripped into both eyes prior to the cotton-tip swab being gently rubbed across the cornea (**Figure 2.8**). Swabs from each animal were deposited in a universal container, sealed with parafilm (Pechiney, Chicago, USA) and stored at -70°C without diluents.

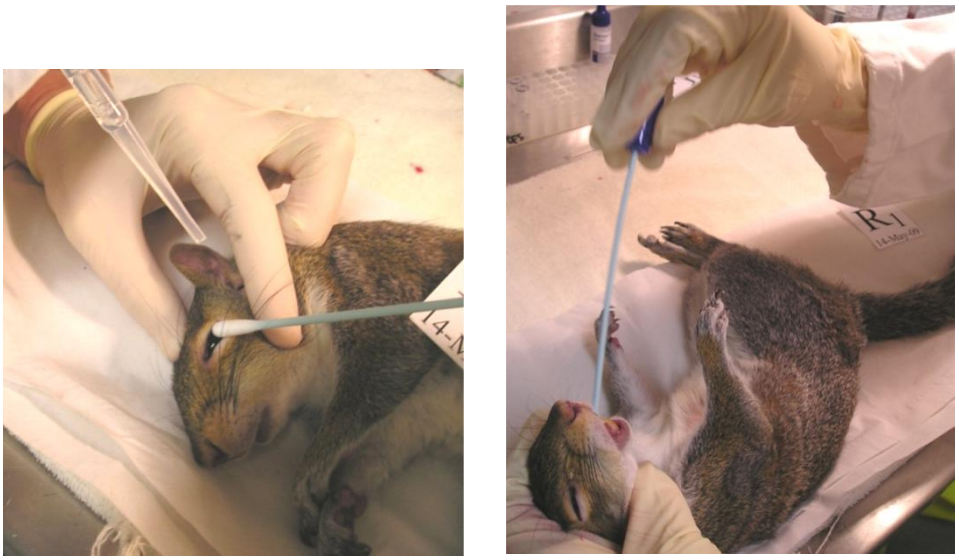


Figure 2.8 Oral and ocular swab.

Example of ocular (left) and oral (right) swab sampling.

2.7 Euthanasia and post mortem procedures

At the end of the experimental infection period, squirrels were euthanized by intracardiac injection of Pentobarbitone sodium B.P. (approx. 200 mg/kg, Rhone Merieux, England). Post mortem examination and procedures were performed immediately thereafter. General external examination was performed with the

estimated age, gender, body condition and characteristic features (e.g. mammary development and scrotal pigmentation) recorded as shown in the protocol used for the post mortem examination (**Figure 2.10**). Biometric measurements were also taken at this stage. Crown-rump length (CRL) and tibial length were taken using a rigid plastic ruler with the animal lying in lateral recumbency. The CRL was measured from the tip of the ear to the last sacral vertebra (**Figure 2.9** green line) with the tail stretched out forming an angle of 90 degrees with the spinal cord. The tibial length was taken by flexing the leg and measuring the distance between the patella and the tibia/fibula-tarso junction (**Figure 2.9** red line).

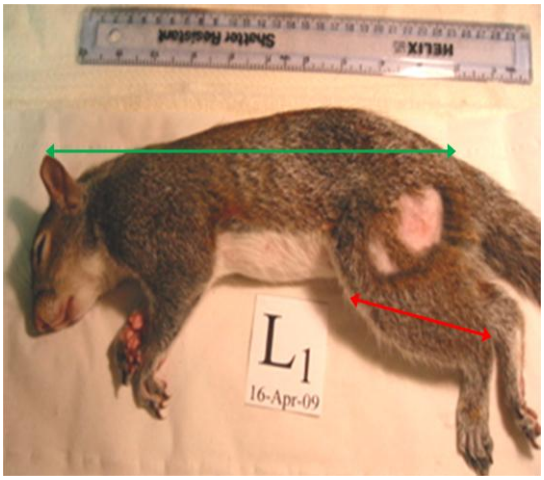
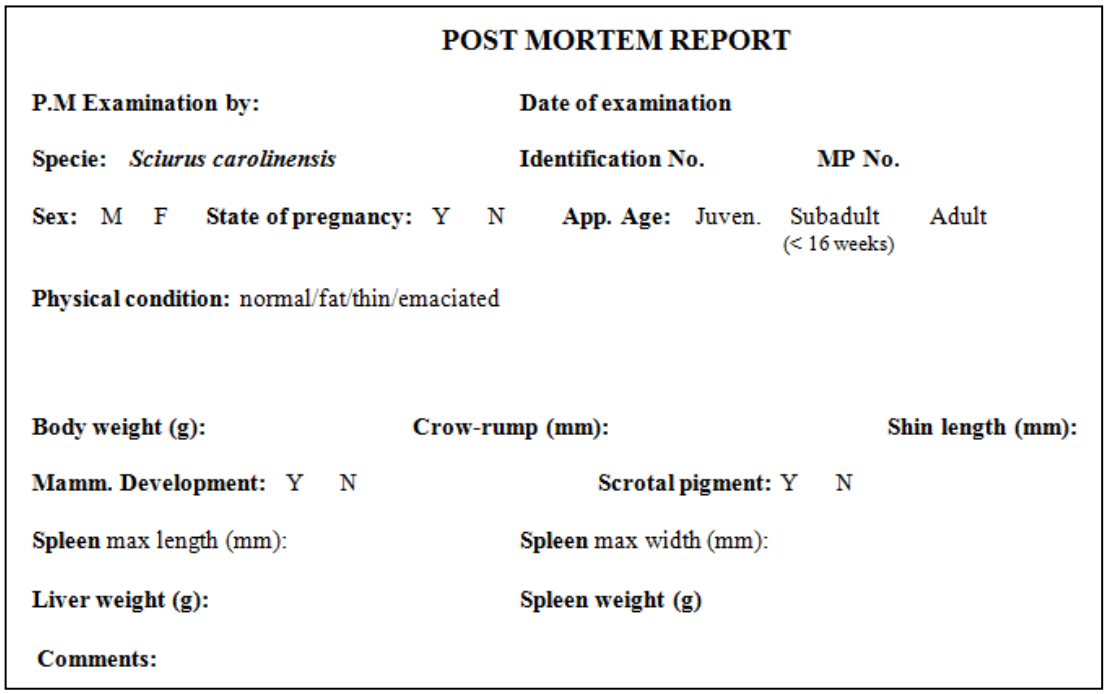


Figure 2.9 Biometric measurements taken post mortem.

The picture illustrates the biometric measurements taken prior to dissection of the carcass. The green line represents the Crown-Rump Length from the tip of the ear to the last sacral bone. The red line represents the tibia length, measured from the patella to the distal end of the tibia-fibula articulation with the tarsal bone.



POST MORTEM REPORT

P.M Examination by: **Date of examination**

Specie: ***Sciurus carolinensis*** **Identification No.** **MP No.**

Sex: M F **State of pregnancy:** Y N **App. Age:** Juven. Subadult (< 16 weeks) Adult

Physical condition: normal/fat/thin/emaciated

Body weight (g): **Crow-rump (mm):** **Shin length (mm):**

Mamm. Development: Y N **Scrotal pigment:** Y N

Spleen max length (mm): **Spleen** max width (mm):

Liver weight (g): **Spleen weight (g)**

Comments:

Figure 2.10 Post mortem report.

Post mortem report sheet used for each squirrel.

After the general external examination was completed, samples were collected for subsequent molecular and histochemical analyses.

Scarified skin samples were collected first to avoid cross-contamination. Other representative skin samples were collected next. Submandibular lymph nodes (SM LN), palatine tonsils and tongue were collected in sequence followed by the submandibular salivary glands and parotid salivary glands. All major body organs were examined for evidence of macroscopic abnormalities followed by duplicate slicing of approx. 0.5 cm sections for processing. When organs were too small to be divided and if they were in pairs (e.g. palatine tonsils, SM LN or submandibular salivary glands,) one was collected for histopathology and other one for molecular studies. The popliteal lymph nodes were collected only for molecular analyses. In some cases, additional lymph nodes were collected for analysis if they appeared to be enlarged (e.g. brachial lymph nodes).

Once the rib cage was cut and sternum removed, the heart and lungs were removed from the thoracic cavity. Spleen and liver were removed from the abdominal cavity and, before they were sliced, their weight was recorded and, additionally for the spleen, length and width were measured as shown in **Figure 2.11**.

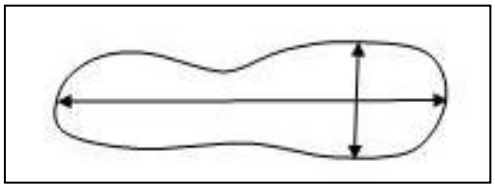


Figure 2.11 Spleen measurements.

Maximum spleen length (horizontal line); Maximum width (vertical line) measurements.

2.8 Histology

Representative pieces of selected tissues were fixed for a minimum of two days to a maximum of six weeks in 10% formal saline. After fixation, tissues were processed overnight, using an automatic processor. Briefly, tissues were dehydrated first by using graded methylated spirit industrial (74 O.P., Fisher Scientific, UK) solution and then cleared in graded xylene solutions before being transferred to a bath of paraffin wax. Samples were oriented and embedded in paraffin wax in a plastic mould. After the wax was completely solidified, the cassette was removed from the mould and 4 micron (µm) thick slices were cut using a microtome, then floated onto a water bath and mounted onto a glass slide. The sections were allowed to dry overnight then stained using an automatic stainer: sections were dewaxed in xylene then taken to water through 74 O.P. (Fisher Scientific, UK) and stained with haematoxylin, washed in water, blued in Scott's tap water substitute (STWS; see **Appendix 1.0**), washed with water again, then counterstained with eosin before being dehydrated using 74 O.P., cleared in xylene and mounted with a synthetic mountant. Sections were examined by light microscopy after staining.

2.9 SQPV Immunohistochemistry

A biotinylated primary antibody and a streptavidin-biotin affinity system was used for the detection and localization of SQPV antigen in skin samples.

Selected blocks of formalin–fixed, paraffin wax-embedded skin tissue (as explained in **§2.8**) were subjected to immunohistochemistry. Duplicate 5 µm thick sections from each block were mounted on glass microscope slides (Superfrost Plus; Menzel-Gläser, Braunschweig, Germany) and allowed to dry at 60°C for one hour and then at 37°C overnight. Sections were dewaxed in xylene, rinsed in 100% v/v methylated spirit industrial (74 O.P., Fisher Scientific, UK) and then placed in 95% v/v 74 O.P. before endogenous peroxidase activity was blocked with 3% v/v hydrogen peroxide (H_2O_2) in methanol (Fisher Scientific) for 20 min. The slides were washed in tap water and then transferred into warmed water for 2 min prior to antigen retrieval (AR) which was done by placing the slides in tris-EDTA buffer (10mM Tris Base, 1mM EDTA, 0.05% Tween20, pH 9.0, see Appendix 1.0) for 10 min at 95°C. This step was necessary as formalin fixation can result in protein cross-linking masking the antigenic sites in the tissue therefore giving false negative results for detection of virus antigen (**Figure 2.12** no.1). After AR the slides were allowed to cool to 50°C before being washed in tap water for five min. Coverslips (Thermo Shandon) were applied to the slides and then were inserted into a Sequenza chamber (Thermo Shandon) before being washed twice in phosphate buffered saline containing 0.05% $Tween_{20}$ v/v (PBST).

As endogenous biotin, a water soluble B-complex vitamin, is present in many tissues, a pre-incubation step with streptavidin, a protein purified from *Streptomyces avidinii* with a high affinity for the biotin molecule, was used to block the binding sites present on the endogenous biotin (**Figure 2.12** no.2). Slides were treated with 100 µl of StreptAvidin (StreptAvidin/Biotin blocking kit, Vector® Labs. CA, USA) for 15 min then washed with PBST. A further incubation step of 15 min with biotin solution (StreptAvidin/Biotin blocking kit, Vector® Labs. CA, USA) was used in order to block all the additional binding sites present on the StreptAvidin molecules (**Figure 2.12** no.3).

Tissue sections were then treated with 100µl of either biotinylated IgG purified from pools of SQPV ELISA positive sera (final concentration of IgG = 16µg/ml diluted in PBST) or biotinylated IgG purified from pools of SQPV ELISA negative grey squirrel sera (final concentration of IgG = 17 µg/ml diluted in PBST) (prepared previously by David Deane, Moredun Research Institute, Scotland, UK) (**Figure 2.12** no.4). Slides were incubated overnight at 4°C prior to being washed three times with PBST. StreptAvidin horseradish peroxidise (HRP) enzyme complex (diluted 1/1000 in PBST, Abcam, UK) was added for one hour at room temperature (RT) (**Figure. 2.12** no.5) then sections were washed as before and the chromogen NovaRED™ (NovaRED™, Vector® Labs., CA, USA) applied as per the manufacturer's instructions (**Figure 2.12** no.6).

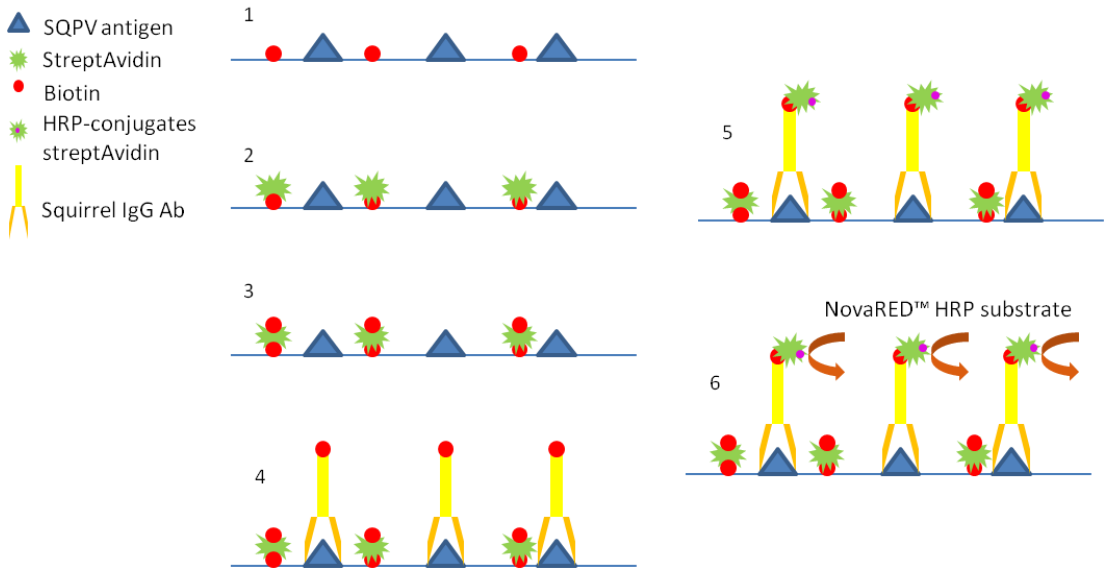


Figure 2.12 Diagram illustrating the SQPV immunohistochemistry assay.

Step 1: Section after antigen retrieval treatment; exposed viral antigen and presence of endogenous biotin in the tissue sample. Step 2: StreptAvidin solution is added and interaction with biotin molecules occurs rapidly. Step 3: incubation with biotin solution to block all the additional binding sites still present in the StreptAvidin proteins. Step 4: Incubation of biotinylated polyclonal IgG antibody from SQPV ELISA positive grey squirrels which binds to SQPV antigen. Step 5: Addition of HRP-conjugated StreptAvidin solution. StreptAvidin binds to the biotin molecules on the IgG and the conjugated HRP provides enzymatic activity for the NovaRed colour substrate as shown in step 6.

Tissue sections were removed from the Sequenza system and washed in tap water then counter-stained with haematoxylin for 2 min, washed in tap water and blued in Scott's tap water substitute (STWS). After thorough washing in running tap water, slides where dehydrated through graded alcohols from 70%, 95% and 100% v/v, cleared and mounted using Consul–Mount (Thermo Scientific, Cheshire, England).

CHAPTER 3

NATURALLY OCCURRING SQPV DISEASE IN WILD RED AND GREY SQUIRRELS IN THE UK

For the post mortem examination data in this chapter I am grateful to staff of the Royal (Dick) School of Veterinary Studies within the University of Edinburgh who performed the post mortem examinations and allowed me to have access to the reports for 11 cases of disease in red squirrels. Special thanks to Prof. Elspeth Milne and Anna Meredith. For cases R597/07, R811/08 and R812/08 I am grateful to Dr. Mark P. Dagleish, Moredun Research Institute, who provided the post mortem reports. Thanks also to Janice Gilray and her staff from Moredun Research Institute for providing the ELISA results for this chapter.

3.1 Introduction

Outbreaks of SQPV-associated disease in red squirrels occur only in areas also inhabited by grey squirrels that are seropositive for SQPV (Rushton *et al.*, 2006; Sainsbury *et al.*, 2000). Manifestation of the infection in the two species, however, is very different. In the red squirrel SQPV disease presents as an exudative erythematous dermatitis (Tompkins *et al.*, 2002; Thomas *et al.*, 2003; Scott *et al.*, 1981a; Sainsbury & Ward, 1996; Sainsbury & Gurnell, 1995). Haemorrhagic crusts typically cover the area around the mouth, periocular area, digital skin, medial skin of the limbs, ventral and genital regions. Ulceration and secondary infection of the lesions also appears typical. However, no significant gross abnormalities suggestive of a viral infection are present in the internal organs (Tompkins et al., 2002) although the vast majority of red squirrels exposed to the virus are likely to die from the disease (Tompkins *et al.*, 2002; Thomas *et al.*, 2003; Sainsbury & Ward 1996; Carroll *et al.*, 2009).

In contrast, SQPV appears to be carried asymptomatically by the grey squirrels which are regarded as the reservoir host for the virus (Sainsbury *et al.*, 2000;

Tompkins *et al*., 2002). The serological status of the two species is also very different; approximately 61% of grey squirrels in England and Wales have antibodies to SQPV whereas relatively small numbers of red squirrels (2-3%) have antibodies to the virus and all the seropositive animals were found dead or dying with the signs of disease (Sainsbury *et al*., 2000).

Detection of disease in squirrels was originally (and still is) based on the observation of clinical signs; this was followed by the use of electron microscopy (EM) which allowed unambiguous identification of the infectious agent as a poxvirus (Scott *et al*., 1981a). In 2000 a SQPV ELISA test was developed in order to detect antibodies against SQPV in squirrel sera. Although these tests have successfully been used to study SQPV spread and prevalence in grey and red squirrel populations, (Sainsbury *et al*., 2000; Sainsbury *et al*., 2008) both EM and the ELISA have limitations: EM has a sensitivity limit of approximately 10^6 particles (Roingeard, 2008) and virus levels below this concentration are not easily detected; serology can detect SQPV antibody in those animals that have contracted the infection, however, there may be animals that are ELISA negative because they are in the early stages of disease but are infectious or conversely animals could test positive but no longer be infectious.

In 2007, two years after SQPV infection of grey squirrels emerged in the south of Scotland, outbreaks of disease in red squirrels were reported (McInnes *et al*., 2009). Since nothing was known definitively about the replication of SQPV in red or grey squirrels, the opportunity was taken to examine these diseased red squirrels along with grey squirrels from the same region that had been culled as part of a government-sponsored grey squirrel control programme. Post mortem examinations were performed on both red and grey squirrels and tissue samples taken for assessing the presence or absence of SQPV DNA. This required optimisation of a quantitative polymerase chain reaction (qPCR) assay before determining in which tissues viral DNA could be detected and quantified.

The aim of this chapter were therefore focused on validating a Real-time PCR TaqMan® assay (qPCR) to detect SQPV DNA in squirrel tissues and compare the presence of SQPV DNA in red and grey squirrel tissues from both ELISA- negative and ELISA-positive animals. Additionally, these results were considered in the wider

context of the overall findings from the post mortem and histopathology examination of the squirrels.

3.2 Naturally occurring squirrelpox in wild red squirrels

3.2.1 Post Mortem findings

Red squirrel carcases found by field workers, red squirrel conservation organizations, ranger services, members of the public and veterinary practices were submitted to the Royal (Dick) School of Veterinary Studies at the University of Edinburgh or to the Moredun Research Institute (red squirrels R597/07, R811/08 and R812/08). Where the condition of the carcasses permitted, a full post mortem investigation was undertaken. When possible, all the squirrels were also tested for the presence of SQPV antibodies. If the condition of the carcass did not permit a blood sample to be taken, cavity fluid was obtained instead for the serological tests. Not all the animals with disease died naturally; some were found alive but in stressed condition and euthanasia was performed by a veterinary practitioner or ranger.

Pathological examination of red squirrel carcasses (with the exception of R597/07, R811/08 and R812/08) followed the protocol of LaRose *et al*., 2010. Briefly, the following information was documented; the squirrel's history, including the grid reference of the site where the animal was found; if it was submitted from a veterinary practice or other organisation; morphological and biological data e.g. gender, estimated age, state of sexual development, body length and weight; the presence of external parasites and the degree of infestation. When possible, external parasites were also collected for qPCR detection of SQPV DNA. Macroscopic lesions were recorded to establish the possible cause of death. When SQPV disease was not suspected (R05/08), tissues samples collected were generally confined to eyelid, digital and lip skin and when SQPV disease was suspected additional samples from different tissues were collected for molecular and histological studies (**Table 3.6**). A skin lesion sample was also taken from suspected SQPV-infected animals for confirmation of SQPV presence by EM. **Figure 3.1** illustrates the typical clinical lesions of SQPV in a diseased red squirrel (R597/07).

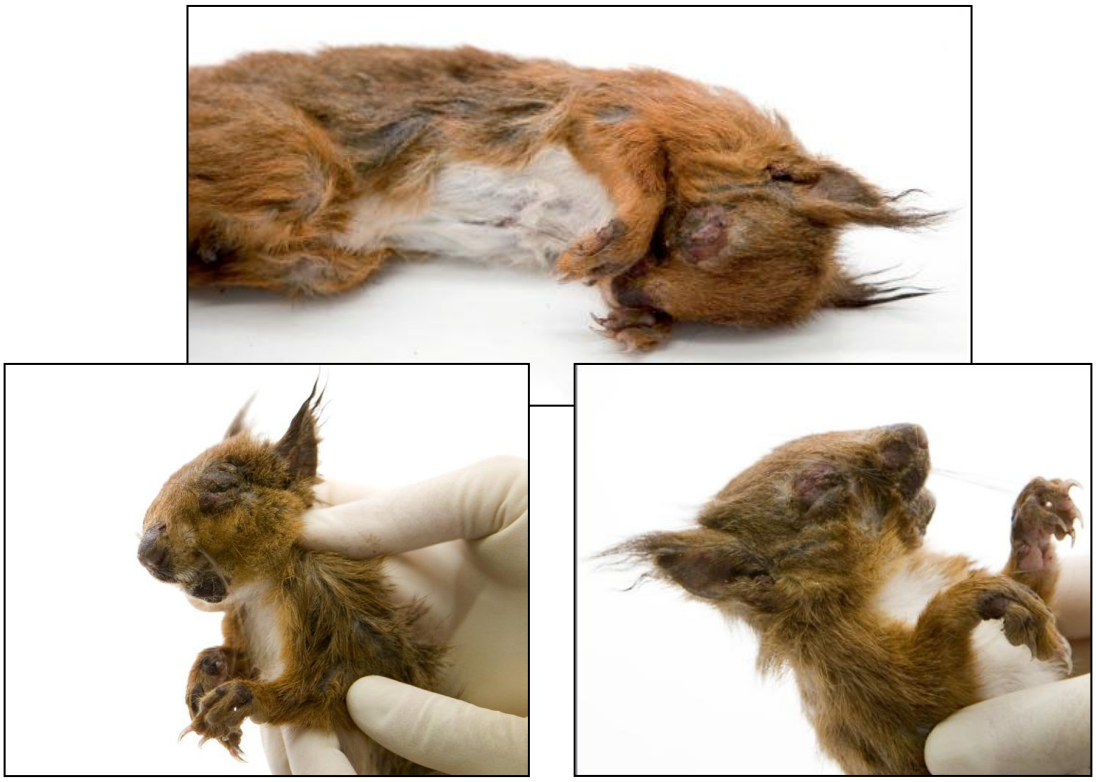


Figure 3.1 Lesions associated with SQPV infection in red squirrels (*Sciurus vulgaris*).

Red squirrel case R597/07. The three pictures show the clinical appearance of SQPV disease in red squirrel with crusty erythematous alopecia and lesions present mostly on the eyelids, nose, lips, digital and footpad skin. Some minor lesions are also present on the pinnae.

Generally in SQPV infected red squirrels lesions were covered by haemorrhagic crusts and mainly covered the skin of the nose, lips, pinnae, digits and the periocular region of both eyes. All the animals in this part of the study had similar lesions to a greater or lesser extent, with the exception of R05/08 which had no obvious skin lesions. A general summary of the lesions and their location on the body reported during the post mortem examinations is given in **Table 3.1**.

Table 3.1 Summary of location and characteristics of external lesions found in 14 red squirrels

Squirrel	Presence of lesions							
	Periocular	Nose	Lips	Ear	Fore foot	Hind foot	Prepuce, scrotal, groin skin	Other skin site
R597/07	2+*	*	*+	+	+	+		*
R21/07	2+	+		+	+*	+	*	+
R23/07	2+	+	+		+			+
R52/07	1*		*		*	*	*	*
R69/07		+	+		*	+	+	
R86/07	1^0		*					
R05/08			*					
R06/08	1*		+				*	*
R27/08	2*		+				*	
R811/08	1+							
R812/08	2+	+	+					
R23/09	2+	+	+		+	+		
R24/09	$1+^0$	+	$+^0$		+			+*
R31/09	2*		*					

(1) Unilateral lesion; (2) Bilateral lesion; (+) Presence of scabs; (*) Ulcerated or excoriated lesion; (^{0}) Purulent exudation.

Of particular interest was the bilateral symmetry of lesions in some squirrels, particularly the peri-ocular lesions, whereas in other animals (e.g. R52/07; R86/07) lesions were recorded on only one side of the body.

Table 3.2 provides an overview of the gender, estimated age, post mortem findings and EM results of the 14 red squirrels examined, illustrating the consistency in external SQPV lesions. Other than the skin lesions there were no external abnormalities that were suggestive of a common pathological agent and, equally, there was a general absence of gross pathology indicative of a viral infection. Several red squirrels in this study were found with ingesta present in the stomach and intestine indicating that the animal had eaten before it died.

Table 3.2 Summary of the post mortem findings from 14 red squirrels

Case	SEX	Age	EXTERNAL LESIONS	GROSS PATHOLOGY	SQPV Suspected
R597/07	♂	A	Thin body condition. Excoriation around both eyes and muzzle. Scabs presence on all manus and feet; more severe on front-feet.	White mineral deposits were present adjacent to the left kidney and also in the corttex of the latter. The stomach was full. Presence of faecal matter in caecum and rectum. Lungs appeared mottled	Y
R21/07	♂	A	Thin body condition. Scabby lesions: periorbitally, both ears, nose, both manus with crusting and ulceration on left hind, medial aspect and inter-digital skin. Thick crusts on flanks, groin, prepuce and scrotum: exudation and ulceration	Froth in lower airways. Adequate ingesta in stomach and intestine.	Y
R23/07	♀	A	Normal body condition. Scabs present: periorbitally (plus alopecia), forefeet and left hip and around mouth and nose. Lips swollen and scabbed.	Submandibular lymph node moderately enlarged. Adequate ingesta in stomach and intestine. Pelleted faeces in colon.	Y
R52/07	♂	A	Thin body condition. Exudative, excoriated skin lesion from axilla to groin on left side, approx 12.5 x2 cm. Similar lesions around left orbit,left upper and lower lip, left carpus and left foot	Patchy congestion of right lung and depressed areas on left dorsal lung.	Y
R69/07	♂	S/A	Thin body condition. Scabby lesions around lips, nose and chin. Scabs on forepaws, right axilla, groin and plantar aspect of right hind foot.	Gas in the intestine, scent ingesta.	Y
R86/07	♂	A	Normal body condition. Left eyelid purulent, left upper lip indented lesion with necrotic tissue overlying.	None	S
R05/08	♂	S/A	Thin body condition. Very small eroded areas on upper lips in medial plane just below the nostrils.	Small areas of consolidation right and left antero-ventral and left caudal margin of lungs. Scant ingesta present.	N/S

Table 3.2 (cont.). Summary of the post mortem findings from 14 red squirrels

Case	SEX	Age	EXTERNAL LESIONS	GROSS PATHOLOGY	SQPV Suspected
R06/08	♀	S/A	Thin animal. Left eyelid swollen and ulcerated. Chin swollen and scabby. Multiple areas of ulceration on ventrum, perineal area and over shoulders.	Stomach moderately full.	Y
R27/08	♂	A	Thin animal. Superficial ulceration around both eyes, no scabs. Large scab on lower lip, ulceration both inguinal region plus caudal scrotum.	Stomach contained only fluid. Large intestine full of formed faecal pellets.	Y
R811/08	♂	A	Scab on right eyelid, no fleas or ticks present, unhatched blow-fly eggs in the mouth.	Stomach full and spleen distended (probably due to barbiturate overdose)	Y
R812/08	♂	A	Scabs present round eyes, nose and extending around the lips. Area of alopecia over the pelvic area.	Thoracic cavity contained whole blood. Stomach approx. one quarter full. Rectum contained formed faeces.	Y
R23/09	♀	A	Normal physical condition. Marked scab formation around eyes, nostrils and top of nose, Right forearm, right and left fore digits, right and left dorsal metatarsus.	No abnormalities. Intestine contained no formed faecal pellets.	Y
R24/09	♂	A	Thin/emaciated animal. Severe scabbing and purulent exudation in left periorbital area left upper and lower lip, chin and top of nose, all digits of forefeet, medial thigh, and right hock with ulceration also affecting the head lesions.	No internal abnormalities.	Y
R31/09	♀	A	Normal physical condition. Ulcerated chin, right upper lip and bilaterally in periorbital regions.	Perineal staining with faeces. Ingesta in stomach.	Y

3.2.2 Histopathological examination

Histological lesions in naturally infected red squirrels were multifocal and mainly located in the skin. Skin lesions were generally covered by a thick haemorrhagic crust containing layers of degenerated keratinocytes and polymorphonuclear (PMN) leukocytes forming a grossly visible scab. **Figure 3.2** shows an example of histological examination of a lip skin lesion from an infected red squirrel (R23/07). Histological examination of the lip lesion showed a moderate acanthosis (epidermal hyperplasia), mild spongiosis and a focal area of acantholysis, more extended at the level of the dermo-epidermal junction within the area containing the epidermal erosion. A mild irregularly distributed inflammatory infiltrate was present within the dermis with accumulation of mixed inflammatory cells at the dermo-epidermal junction.

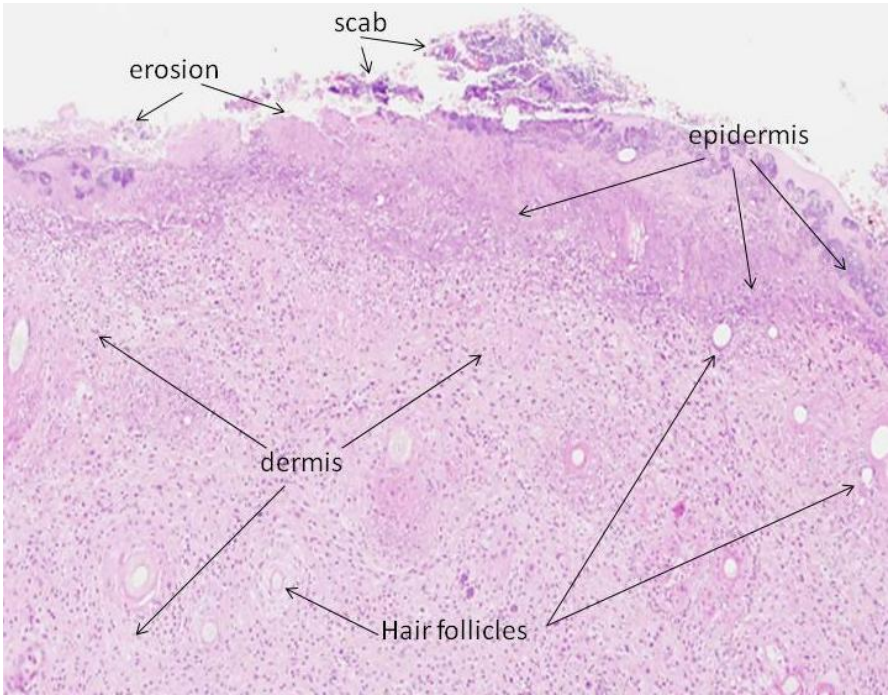


Figure 3.2 Red squirrel lip skin lesion showing scab formation and epidermal erosion.

Red squirrel R23/07. Skin lesion. Histological examination shows a moderate epidermal hyperplasia and within the epidermis the erosion is covered by a moderate serocellular crust (scab) and infiltrated mainly by neutrophils. There is an underlying mixed inflammatory response within the dermis. The inflammatory infiltrate is band-like at the dermo-epidermal junction. Haematoxylin and Eosin x 100.

Generally, microscopic lesions of the skin or tongue of infected red squirrels were similar to the one describe above and usually characterized by a multifocal epidermal hyperplasia of the stratum spinosum (acanthosis) and ballooning degeneration in keratinocytes (**Figure 3.3**). Occasionally, in the degenerated keratinocytes of the stratum spinosum, there were eosinophilic intracytoplasmic inclusion bodies, one per cell, surrounded by a clear halo (**Figure 3.3 B** and **D**). Such inclusion bodies have been described previously in poxvirus infections and are regarded as confirmatory for poxvirus infection.

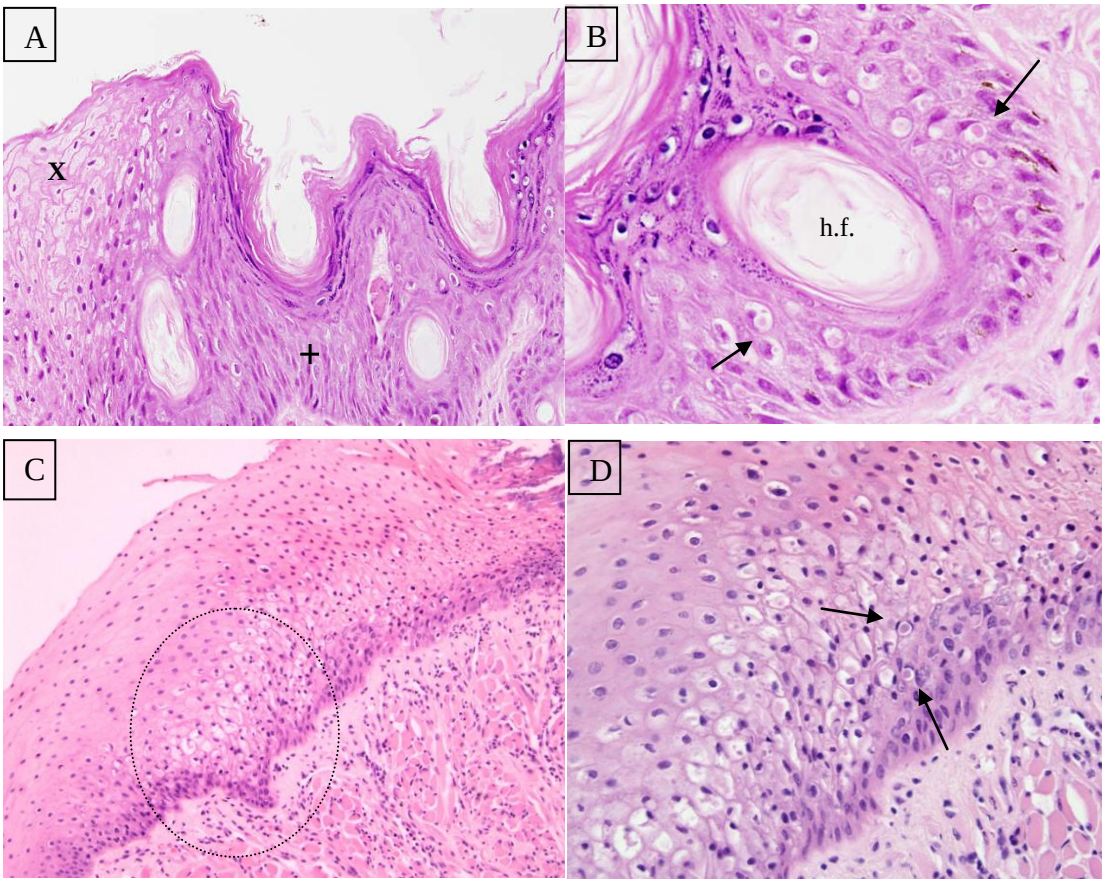


Figure 3.3 Red squirrel (*Sciurus vulgaris*), haired skin and tongue lesion.

(**A**) Haired skin of red squirrel R21/07. Focal epidermal hyperplasia (indicated by +) and ballooning degeneration (indicated by X) of the epidermis. (**B**) Pox-like eosinophilic intracytoplasmic inclusion body (arrows); (h.f.): hair follicle. (**C**) Dorsal aspect of tongue of red squirrel R597/07. Mixed inflammatory cell dermatitis. Area of ballooning degeneration (dashed circle). (**D**) Higher magnification of picture C; note the presence of pox-like intracytoplasmatic amphophilic inclusion body in the epithelium (indicated by arrows). Haematoxylin and eosin (A x 20, B x 40, C x10, D x 40).

3.2.3 PCR assays for detecting SQPV DNA

The polymerase chain reaction (PCR) is a sensitive molecular technique which permits the detection of small amounts of DNA and RNA. Initial analyses of the SQPV cases in Scotland employed the conventional (gel-based) PCR and additionally the TaqMan® Real-time PCR (qPCR) method, to detect SQPV DNA, was refined as part of this study.

Both techniques are based on the same reaction (PCR) but the main difference between the two assays is that the real-time PCR assay, with the inclusion of an additional fluoresecent probe detection system, results in a more sensitive technique and has the ability to confirm and quantitate the target concentration (Ratcliff *et al*., 2007). In the conventional PCR results are analyzed at the end point of the reaction and rely on visualization of fluorescent bands of defined molecular weight after agarose gel electrophoresis. In contrast, the qPCR reaction is followed in "real time" by monitoring the reporter fluorescence during the reaction, resulting in a value which is proportional to the amount of the target PCR product (amplicon). A threshold line is set (**Figure 3.4**) and the point at which the florescence signal from a particular sample crosses the threshold is recorded. This is known as the crossing point (Cp) and is inversely proportional to the initial amount of DNA target in the sample. **Figure 3.4** shows a representation of a qPCR amplification plot and the way it is interpreted compared to conventional PCR.

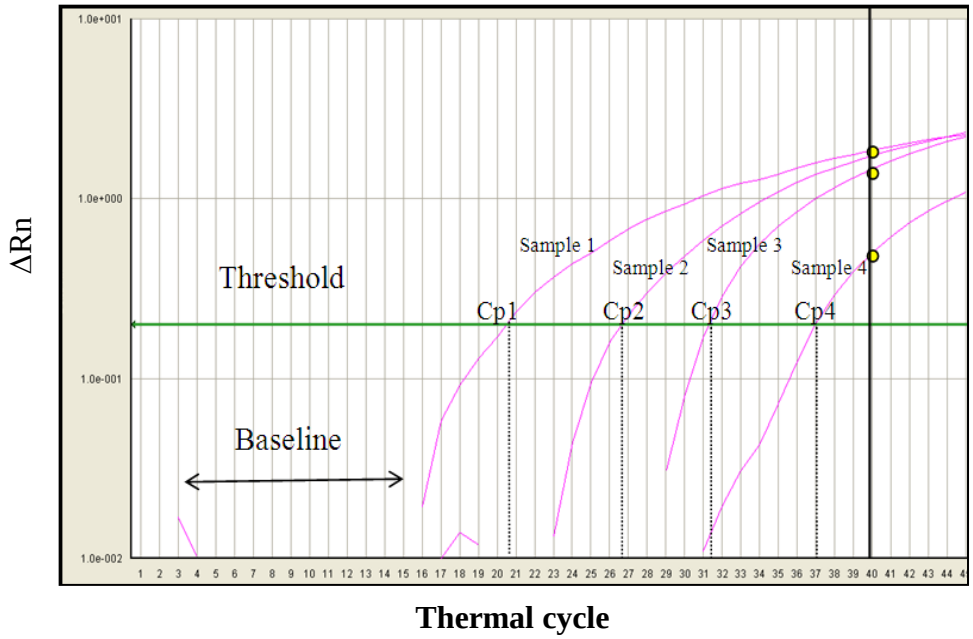


Figure 3.4 Representative qPCR log plot of amplification curves.

qPCR amplification plot (fluorescence signal versus thermal cycle number). The C_p (crossing point) is defined as the thermal cycle point at which the fluorescence of the sample crosses the fixed green line (threshold) which is set on the linear phase of the reaction. In the initial cycle of the qPCR there is an increase in the fluorescence (baseline) which is considered as background noise; the increase in the fluorescence above the baseline is defined as detection and amplification of the target. Earlier detection of an exponential increase in the fluorescence is related to a higher amount of target DNA in the initial sample (1) with a lower C_p value (Cp1). The higher C_p value (C_p4) corresponds to a lower initial DNA target in the sample. The vertical black line, set at the 40th cycle represents the end point analysis for a hypothetical conventional PCR. In a conventional gel based PCR, the fluorescence of samples 1, 2 and 3 would be almost the same, but a weaker fluorescent signal would be detected from sample 4 (yellow dots). In contrast, in qPCR all four positive results (pink lines) have a different C_p (C_p1, C_p2, C_p3, C_p4) value inversely proportional to the amount of initial target DNA.

3.2.3.1 Conventional (gel-based) PCR analysis of squirrelpox virus-infected tissues

The conventional PCR was performed as described by McInnes and colleagues (McInnes *et al.*, 2009). The SQPV A11L gene was amplified as the target gene using primers that were designed to produce an amplicon of 258 bp (**§2.5.3**). This gene has only been found so far within the *Parapoxvirus*, *Molluscipoxvirus* genera and SQPV. The PCR reaction was performed using DNA from vaccinia virus strain Copenhagen (VACV), orf virus (ORFV), pseudocowpox virus (PCPV), bovine papular stomatitis virus (BPSV), sealpox virus (SPV), DNA from the digital skin of a non-infected red squirrel and DNA from four of the suspected cases of squirrelpox. No molluscum

contagiosum DNA was available. DNA from SQPV isolate 1296/99 (Thomas *et al*., 2003) was used as positive control and distilled water as a further negative control. Positive results were only obtained from the suspected SQPV-infected red squirrels and the positive control (**Figure 3.5**). This assay was subsequently used as a rapid method for confirming infection of SQPV disease in red squirrels, since the amplicon could easily be cloned and sequenced (refer to **Chapter 6**).

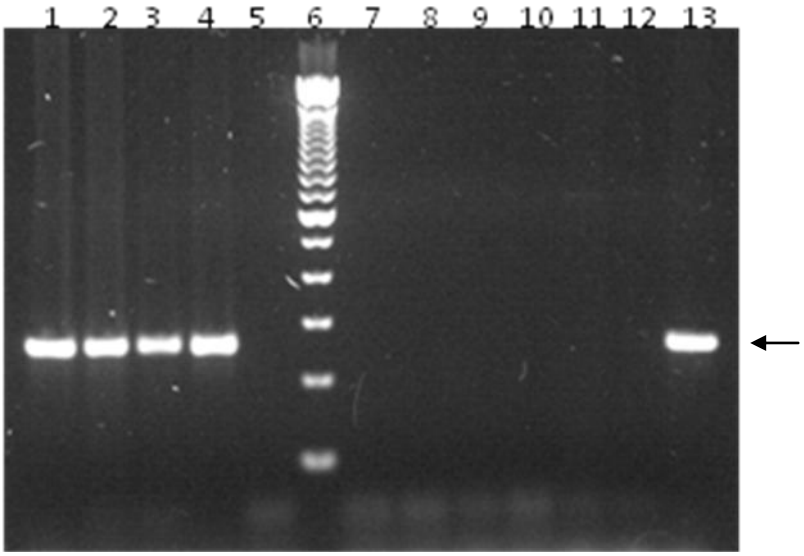


Figure 3.5 Agarose gel based PCR for detection of SQPV.

Agarose gel electrophoresis of PCR amplicons obtained from four SQPV positive red squirrels. From left to right: (1) red squirrel R597/07; (2) red squirrel R21/07; (3) red squirrel R23/07; (4) red squirrel R52/07; (5) ORFV; (6) Ready-loadTM 1Kb DNa Ladder (Invitrogen); (7) BSPV; (8) PCPV; (9)VACV; (10) SPV; (11) SQPV negative red squirrel genome DNA extracted from digital skin; (12) H_2O negative control; (13) red squirrel 1296/99 as PCR positive PCR control.

3.2.3.2 TaqMan® Real-time PCR: standard curve production and optimization of the assays

Two sets of TaqMan® Real-time PCR (qPCR) primers and probes were assessed for detection of SQPV DNA. The assays were based on the amplification of parts of SQPV sequence, in particular the H2L and I1L genes which are present at the left hand (LH) end of the SQPV genome (McInnes *et al*., 2006). The SQPV H2L gene is predicted to encode a RNA polymerase subunit with 59% identity to the Lumpy skin disease virus (LSDV-1956_036) homologue of Vaccinia virus E4L gene. The

I1L gene encodes a possible orthologue of the Molluscum contagiosum virus (MOCV) gene involved in the production of an immunoglobulin domain protein.

To enable an absolute quantitation of the target viral DNA and have reference Cp scores for subsequent tests, initially two qPCR assays were used to detect the appropriate regions from SQPV genomic DNA cloned into a cosmid (SQPV cosmid #86), (Thomas *et al*., 2003). The relative standard curves were established with 10-fold serial dilutions of the cosmid DNA to estimate the dynamic range of quantitation (sensitivity) and efficiency with both target genes.

Briefly, a qPCR master mix (for more details see §**2.5.7**) was prepared and aliquoted into 96-well plates prior to the addition of the SQPV cosmid template. Four replicate amplification reactions were conducted for each concentration of standard DNA, covering seven orders of magnitude. As the SQPV cosmid #86 was serially diluted, bovine genomic DNA was added to obtain a final concentration of 200/µl per reaction in order to maintain the overall DNA concentration in each sample. After completion of the qPCR assays, standard curves were produced using the ABI software (7000 Apply Biosystem software Version 1.2.3) for each of the two targets. Negative samples (bovine genomic DNA) were included in each assay to control for test contamination. The results from the assays are presented in **Figure 3.6**.

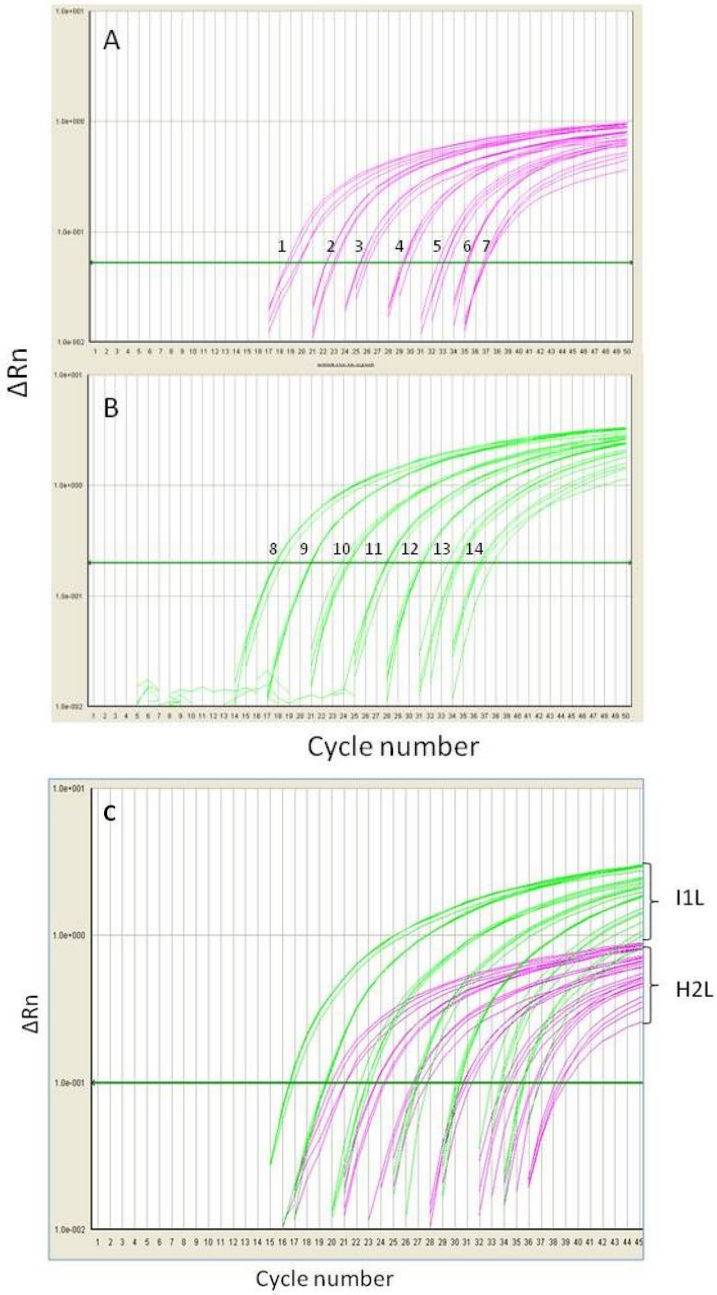


Figure 3.6 Amplification plot for 10-fold dilution series for H2L and I1L gene detectors.

Output of amplification runs used for construction of the standard curves in this study. In all three figures the log of reaction fluorescence is plotted versus thermo cycles (cycle number). Thresholds (horizontal green lines) provide the reference point from which the crossing point (C_p) is calculated. **(A)** qPCR amplification plot targeting H2L gene (pink); 10 fold dilution from a range of $3x10^7$ (1) to 3x10 (7) genomes/equivalent. (**B**) Amplification plot targeting I1L gene (green); 10 fold dilution from a range of $3x10^7$ (8) to 3x10 (14) genomes/equivalent. **(C)** Comparative quantification plot of 10-fold dilution series between H2L and I1L detectors.

Table 3.3 Mean C_p value and standard deviation from amplification runs using two sets of qPCR detectors.

SAMPLE NUMBER	DETECTOR	QUANTITY Genomes equivalent/reaction	MEAN Cp [(a)]	SD [(b)]
1	SQPV H2L	3×10^7	19.2	±0.565
2	SQPV H2L	3×10^6	22.7	±0.461
3	SQPV H2L	300000	25.6	±0.363
4	SQPV H2L	30000	29.5	±0.325
5	SQPV H2L	3000	33.0	±0.409
6	SQPV H2L	300	35.6	±0.732
7	SQPV H2L	30	36.9	±0.086
8	SQPV I1L	3×10^7	15.2	±0.268
9	SQPV I1L	3×10^6	19.1	±0.089
10	SQPV I1L	300000	21.6	±0.308
11	SQPV I1L	30000	25.4	±0.345
12	SQPV I1L	3000	28.6	±0.165
13	SQPV I1L	300	31.9	±0.536
14	SQPV I1L	30	34.4	±0.486

([a]) Mean C_p value corresponding to qPCR standard curve using both H2L and I1L specific primers and probes to amplify a fragment of SQPV DNA derived from SQPV cosmid #86. Four replicate samples were used for each dilution for both detectors.
([b]) SD = Standard deviation from each replicate data set.

The reproducibility of qPCR was estimated using the standard deviation in Cp values generated from replicate amplifications for each set of dilutions for both target detectors. Based upon the standard deviation produced from each individual dilution (**Table 3.3**), the estimated intra-assay variation was lower for the I1L detector (ranging from 0.089 to 0.536) compared to H2L detector (ranging from 0.086 to 0.732). It was concluded that the Cp values obtained for both target genes displayed a satisfactory level of reproducibility over seven magnitudes of target DNA concentration. **Table 3.4** shows the comparison in mean Cp values between the two assays for each of the dilutions of cosmid DNA. The I1L assay produced a lower Cp score than the H2L assay, with the same starting amount of target DNA. The

difference in Cp scores for each dilution ranged from 2.5 to 4.4 with an average of 3.75.

Table 3.4 Comparison between C_p values of the two qPCR detectors used to produce standard curves.

Genomes equivalent/reaction	MEAN Cp		ΔCp H2L& I1L
	H2L	I1L	
3×10^7	19.2	15.2	4.0
3×10^6	22.7	19.1	3.6
300000	25.6	21.6	4.0
30000	29.5	25.4	4.1
3000	33.0	28.6	4.4
300	35.6	31.9	3.7
30	36.9	34.4	2.5

Delta value (ΔC_p) resulting from the mean C_p values generated from replicate amplifications for each set of dilutions for both target detectors (H2L and I1L).

Linear regression analysis was also used to determine the slopes and the intercepts of the two assays. These correspond respectively to the amplification efficiency and the number of the amplicon molecules at the threshold (Rutledge & Côté 2003).
Standard curves generated with the H2L and H1L detectors are shown in **Figure 3.7**.

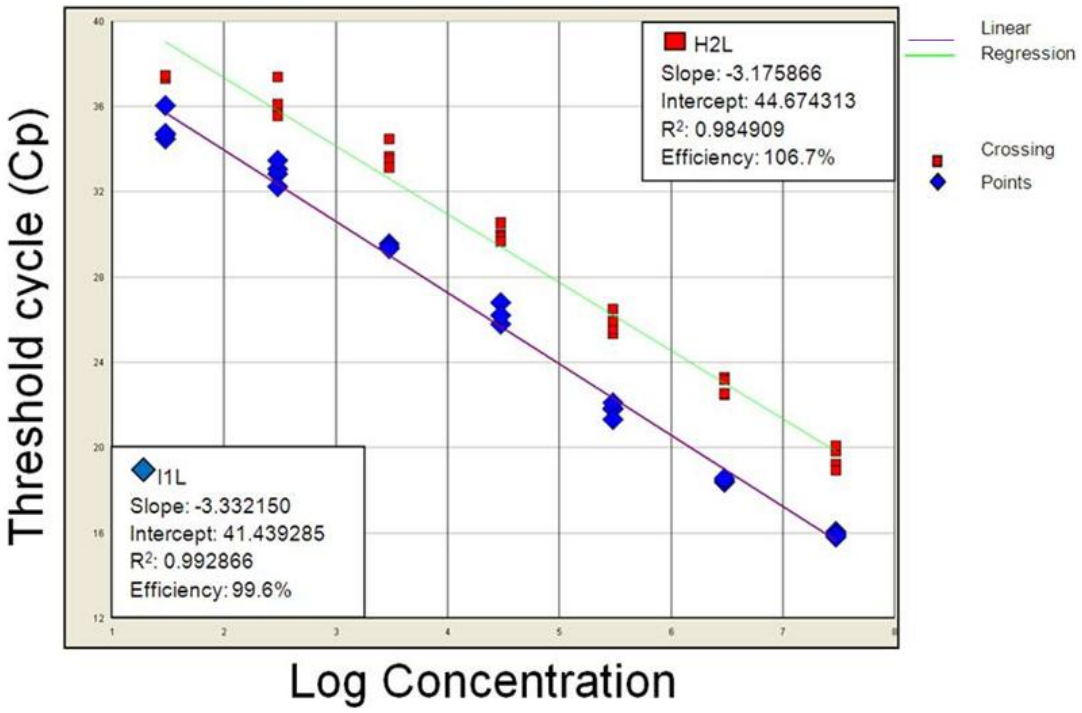


Figure 3.7 Standard curves generated for H2L and I2L genes.

Resulting standard curves generated by plotting the log of the target DNA concentration (Log concentration) against the threshold cycle (C_p) value. The fluorescence of the standard curves is based on the serial 10 fold dilution of SQPV cosmid #86 using the I1L and H2L target genes. The standard curves were analyzed to determine the sensitivity and efficiency of the PCR reaction. The slope of the resulting trend line is given as a function of the PCR efficiency.

The percent of amplification efficiency (E%) for each of the assays was calculated using the slope of the resulting trend line (Rutledge & Côté 2003).

The gradient of the two standard curves was calculated as -3.176 for H2L (green line) and -3.332 for I1L detector (blue line) which are translated into efficiency assay using the following formula:

$$\mathbf{E\% = [10^{(-1/slope)} -1] \times 100}$$ (Rasmussen 2001)

Efficiency for H2L qPCR reaction was calculated as 106.7% and efficiency of 99.6% for I1L assay.

Both assays were able to reliably detect as few as 30 copies of the target sequences (30 genome equivalents); the coefficient of determination (R^2) was 0.985 for the H2L assay and 0.993 for the I1L assay. These results are summarised in **Table 3.5**.

Table 3.5 Summary of the linear regression analysis generated from the standard curves.

DETECTOR	(R^2) VALUE	EFFICIENCY (E%)
H2L	0.98	106.7%
I1L	0.99	99.6%

Results of correlation coefficient (R^2 coefficient of determination) and qPCR efficiency (E%) from the standard curves generated by employing the successively diluted copy number of SQPV cosmid #86 using the two SQPV detectors (H2L and I1L respectively). qPCR efficiency was calculated from the slopes of the given standard curves.

It was considered impractical to perform both the H2L and I1L assays on every occasion that squirrel tissues were to be examined and therefore one assay was selected on the basis of the results obtained from the optimization experiments. While both assays were capable of detecting an equal input of target DNA, the I1L assay routinely gave C_p values approximately four cycles less than the H2L assay (**Table 3.4**) and had a signal amplitude approximately two times higher than that of the H2L assay. The efficiency of the I1L assay was also nearer to the 100% optimum. As a result the I1L assay was chosen as the main assay for subsequent analyses.

3.2.3.3 qPCR analysis of red squirrel tissues

Tissues taken from the fourteen red squirrels subjected to post mortem examination, with the exception of R597/07, were processed for qPCR analysis and are listed in **Table 3.6**.

Table 3.6 Table summarising red squirrel tissues used for the SQPV qPCR study.

Squirrel ID	R21/ 07	R23/ 07	R52/ 07	R69/ 07	R05/ 08	R06/ 08	R86/ 07	R27/ 08	R811/ 08	R812/ 08	R23/ 09	R24/ 09	R31/ 09
Scab	√		√			√		√			√	√	√
Dig. Skin	√	√		√	√	√	√	√	√	√	√	√	
Lip skin	√	√	√	√	√	√	√	√	√		√	√	√
Eye. skin	√	√	√	√	√	√	√	√	√	√	√	√	√
No. skin	√	√	√	√		√	√	√			√	√	√
Tongue	√	√	√	√		√	√	√			√	√	√
Stomach	√	√	√	√		√	√	√			√	√	√
S.I	√	√	√	√		√	√	√			√	√	√
L.I	√	√	√	√		√	√	√			√	√	√
Caecum	√	√	√	√		√	√	√			√	√	√
Rectum	√	√	√	√		√	√	√			√	√	√
SM LN	√	√	√	√		√	√	√			√	√	√
Spleen	√	√	√	√		√	√	√	√		√	√	√
Liver	√	√	√	√		√	√	√	√		√	√	√
Heart	√	√	√	√		√	√	√			√	√	√
Kidney	√	√	√	√		√	√	√			√	√	√
Brain	√	√	√	√		√	√	√			√	√	√
Adr.gland	√	√	√	√		√	√	√			√	√	√
Faeces													√

Top row: squirrel identification (ID). First column: samples origin.
Dig. Skin: digital skin; Eye. Skin: eyelid skin; No. Ski: nose skin; S.I: small intestine; L.I: large intestine; SM LN: submandibular lymph node; Adr. Gland: adrenal gland.

Figure 3.8 contains examples of the qPCR amplification plots produced from the various tissue samples from squirrelpox virus infected red squirrels R23/09 and R31/09.

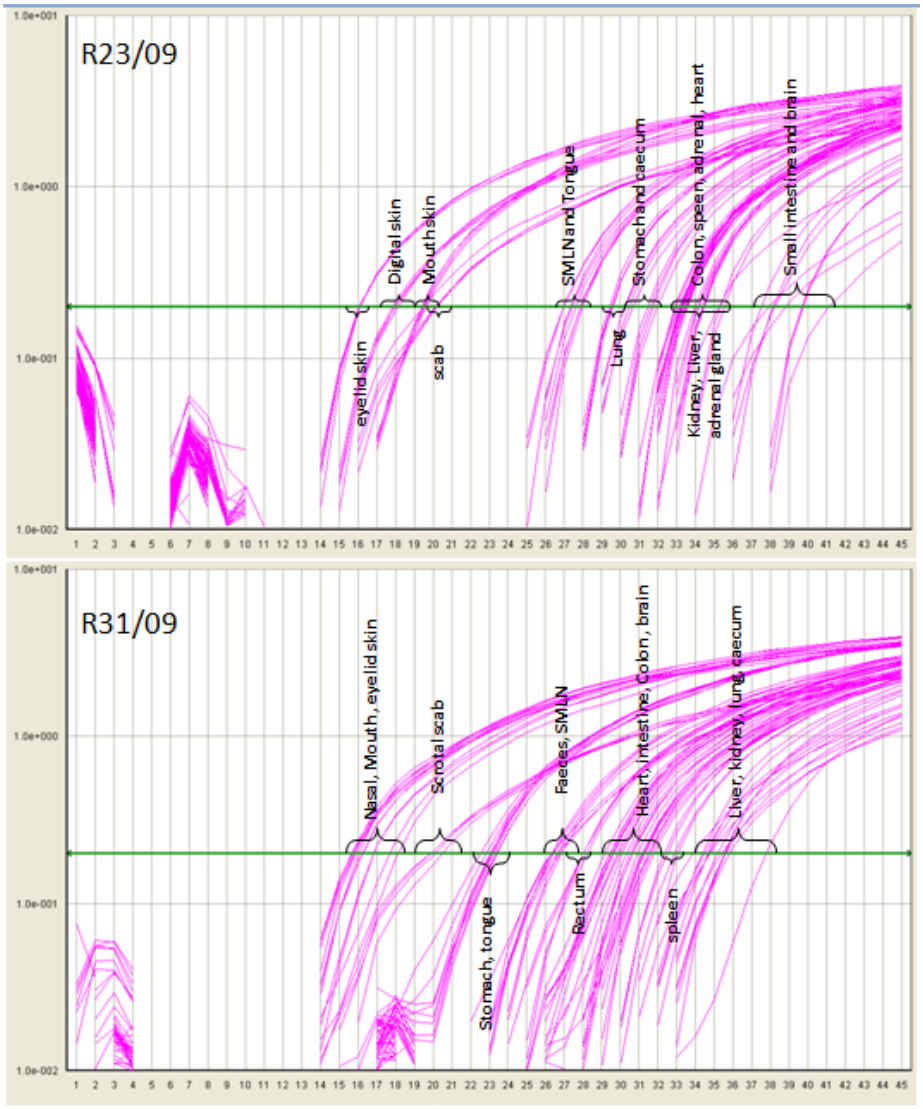


Figure 3.8 Amplification plot of SQPV DNA from squirrel tissues using the optimized I1L TaqMan assay.

Two examples of qPCR amplification plot; fluorescence signal versus PCR cycles number indicating the presence of viral DNA in the different squirrel tissues (see labels in curly brackets). Note that in both animals (R23/09 and R31/09) higher amounts of viral DNA were detected in skin samples. For squirrel R31/09 faeces were also sampled in addition to the standard sample collection. Each sample was run with four replicates. SQPV DNA was detected in almost all the samples analyzed. The qPCR analyses were performed using the protocol for the SQPV I1L gene outlined in §**2.5.7**.

In **Table 3.7** the mean Cp scores for the various tissues analyzed from each individual are summarized along with the serology results. EM results are shown in the bottom row. Absolute quantitation of SQPV DNA was not performed as the information sought was the relative quantity of SQPV present in each tissue. The mean Cp values could, however, be converted into amount of product (genome equivalents) using the standard curve results in section §**3.2.3.2**.

Table 3.7 qPCR mean C_p score results from different tissue samples from SQPV ELISA positive and ELISA negative diseased red squirrels.

Squirrel ID	R21/ 07	R23/ 07	R52/ 07	R69/ 07	R86/ 07	R05/ 08	R06/ 08	R27/ 08	R811/ 08	R812/ 08	R23/ 09	R24/ 09	R31/ 09
ELISA	1.38	0.26	1.60	0.21	N	N	N	N	N	N	1.50	2.96	N
Gender	M	F	M	M	M	M	F	M	M	M	F	M	F
Tissue	**Mean Cp values**												
Scab	15.7	nd	17	ns	ns	ns	20.1	27.2	ns	ns	19	15.2	20.1
Digital skin	28.7	25.8	ns	17.2	N	17.3	30.3	17.6	35.1	23.2	18	16.3	ns
Eyelid skin	31.2	26.3	37.4	22.2	N	N	23.7	42.2	20	16.7	16	16.5	17.4
Mouth skin	33.7	23.4	26.3	17.5	N	25.6	29.7	19.2	16.4	ns	19.4	16.2	16
Tongue	38.6	36.9	N	25.4	N	ns	nd	nd	ns	ns	27.6	25	23.2
Stomach	N	N	N	34.9	N	ns	nd	nd	ns	ns	30.4	24.3	22.8
S.I	37.2	N	N	37.8	N	ns	nd	nd	ns	ns	38.3	33.1	30.2
L.I	N	40.2	N	34.3	N	ns	nd	nd	ns	ns	33.1	32	29.6
Caecum	N	N	N	N	N	ns	nd	nd	ns	ns	31.7	29	31.6
Rectum	N	39.6	N	37	N	ns	nd	N	ns	ns	ns	31	28
SMLN	38.1	38.7	N	37.9	N	ns	nd	nd	ns	ns	27.3	28	26.5
Spleen	N	N	N	N	N	ns	nd	37.8	31.3	ns	33	35	32.7
Liver	40.3	41.2	N	37.5	N	ns	nd	35.8	38	ns	34.3	33.9	35
Lung	44.1	N	N	N	N	ns	nd	nd	ns	ns	29.4	29.2	35
Heart	43	N	N	38.7*	N	ns	nd	nd	ns	ns	33.3	29.3	29.3
Kidney	N	N	N	N	N	ns	nd	nd	ns	ns	33.7	35	34.9
Adr. gland	N	N	N	N	N	ns	nd	nd	ns	ns	34.3	30.3	31.2
Brain	42.2*	42.8	N	40.3	N	ns	nd	nd	ns	ns	38.7	30.3	30.8
Faeces	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	26.5
EM	+	+	+	+	-	+	+	+	ns	ns	+	+	+

Results from red squirrel samples analysed by qPCR. Four replicates were conducted for each sample. SQPV antibody values are expressed as OD_{450}. Electron microscopy (EM) results performed from skin or scab samples (courtesy of D. Everest, VLA, Weybridge). (N) qPCR negative result; (ns) no sample collected; (nd) not determined; (*) three out of four positive replicates; (+) EM positive result; (-) EM negative results.

S.I: small intestine; L.I: large intestine; SMLN: submandibular lymph node; Adr. Gland: adrenal gland.

Serological analyses were performed on the blood or body cavity fluid from each squirrel and EM analysis (courtesy of David Everest, VLA, Weybridge) on a sample of skin or scab to confirm exposure to the virus or the presence of SQPV particles, respectively. With the exception of one squirrel (R86/07) in which no virus particles were found, ten red squirrels were confirmed SQPV positive by EM (with the exclusion of squirrels R811/08 and R812/08 for which no skin samples were sent for EM analysis) including one (R05/08) in which squirrelpox infection had not been suspected from the post mortem examination. Skin samples from squirrel R597/07, which was not analyzed in the qPCR study, but had been tested by standard PCR (§**3.2.3.1**), was also EM positive (**Figure 3.8**).

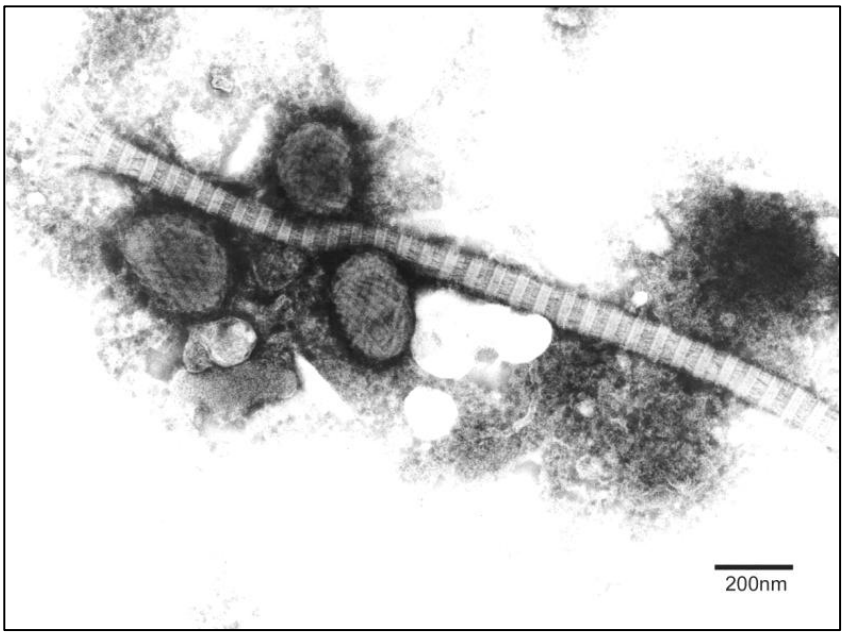


Figure 3.8 Electron microscopy image of SQPV particles; Red squirrel case R597/07.
Courtesy of Mr. David Everest (VLA, Weybridge, UK)

Red squirrels are known to be highly susceptible to SQPV infection and in this study virus was detected by qPCR in almost all of the tissue samples examined. SQPV DNA was always detected in the greatest amounts in scabs, mouth, digital and eyelid skin suggesting that the virus specifically targets the epidermis for its replication (Cp scores generally with a mean of between 19.2 and 29.5). Indeed SQPV DNA was detected in over 91.6% of the samples representing these tissue types (**Table 3.8**).

Table 3.8 Percentage of positive SQPV samples compared with the mean C_p score resulting from qPCR study for 12 naturally infected red squirrels.

Sample	Total samples	% positive	Mean Cp	Sample	Total samples	Mean Cp	% positive
Scab	7	**100**	19.2	**Scab**	7	**19.2**	100
Mouth skin	11	**100**	22.1	**Mouth skin**	11	**22.1**	100
Digital skin	10	**100**	23	**Digital skin**	10	**23**	100
Eyelid skin	12	**91.6**	24.5	**Eyelid skin**	12	**24.5**	91.6
Liver	9	**88.8**	37.0	**Stomach**	7	**28.1**	57.1
SM LN	7	**85.7**	32.8	**Tongue**	7	**29.5**	85.7
Tongue	7	**85.7**	29.5	**Caecum**	7	**30.8**	42.8
Brain	7	**85.7**	37.5	**Adr. gland**	7	**31.9**	42.8
L.I	7	**71.4**	33.8	**SM LN**	7	**32.8**	85.7
Heart	7	**71.4**	34.7	**L.I**	7	**33.8**	71.4
S.I	7	**71.4**	35.3	**Rectum**	7	**33.9**	57.1
Stomach	7	**57.1**	28.1	**Spleen**	9	**34**	55.5
Rectum	7	**57.1**	33.9	**Lung**	7	**34.4**	57.1
Lung	7	**57.1**	34.4	**Kidney**	7	**34.5**	42.8
Spleen	9	**55.5**	34	**Heart**	7	**34.7**	71.4
Caecum	7	**42.8**	30.8	**S.I**	7	**35.3**	71.4
Adr. gland	7	**42.8**	31.9	**Liver**	9	**37**	88.8
Kidney	7	**42.8**	34.5	**Brain**	7	**37.5**	85.7

Results of I1L qPCR assay performed on 18 different tissue samples to detect SQPV DNA from naturally infected red squirrels. Samples are associated with a decreasing percentage of positive results (first grey column) and with a decreasing mean C_p score (second grey column).
Adr. gland: adrenal gland; SM LN: submandibular lymph node; L.I: large intestine; S.I: small intestine.

The tissues that appeared to harbour the next greatest amount of virus were stomach, tongue, caecum, adrenal gland and SM LN but the percent of this positive results were only high (in order 85.7%) for SM LN and tongue, whereas for stomach, caecum and adrenal glands the percent of positive samples was between 57.1% to 42.8%. The single positive faecal sample (Cp 26.5) from squirrel R31/09 was not included in this table since it was the only positive faecal result obtained. Relatively lower levels of viral DNA (mean Cp score 34- 37.5), were detected in the spleen, lung, heart, kidney, heart, small intestine (S.I), liver and brain (**Table 3.8**). Of

particular interest was to note that liver and brain, even if the Cp score was one of the lowest (37 and 37.5), these samples were found resulting positive for the vast majority (88.8%) of samples analysed. The same information is provided pictorially in **Figure 3.10**.

These results are also supported by post mortem findings with the only lesions consistent with SQPV infection being present at skin sites, or tongue, suggesting the virus being epidermotropic. Equally, EM analysis confirmed that there is an abundance of virus particles in epidermal scabs and skin samples whereas no virus particle were ever described in internal organs.

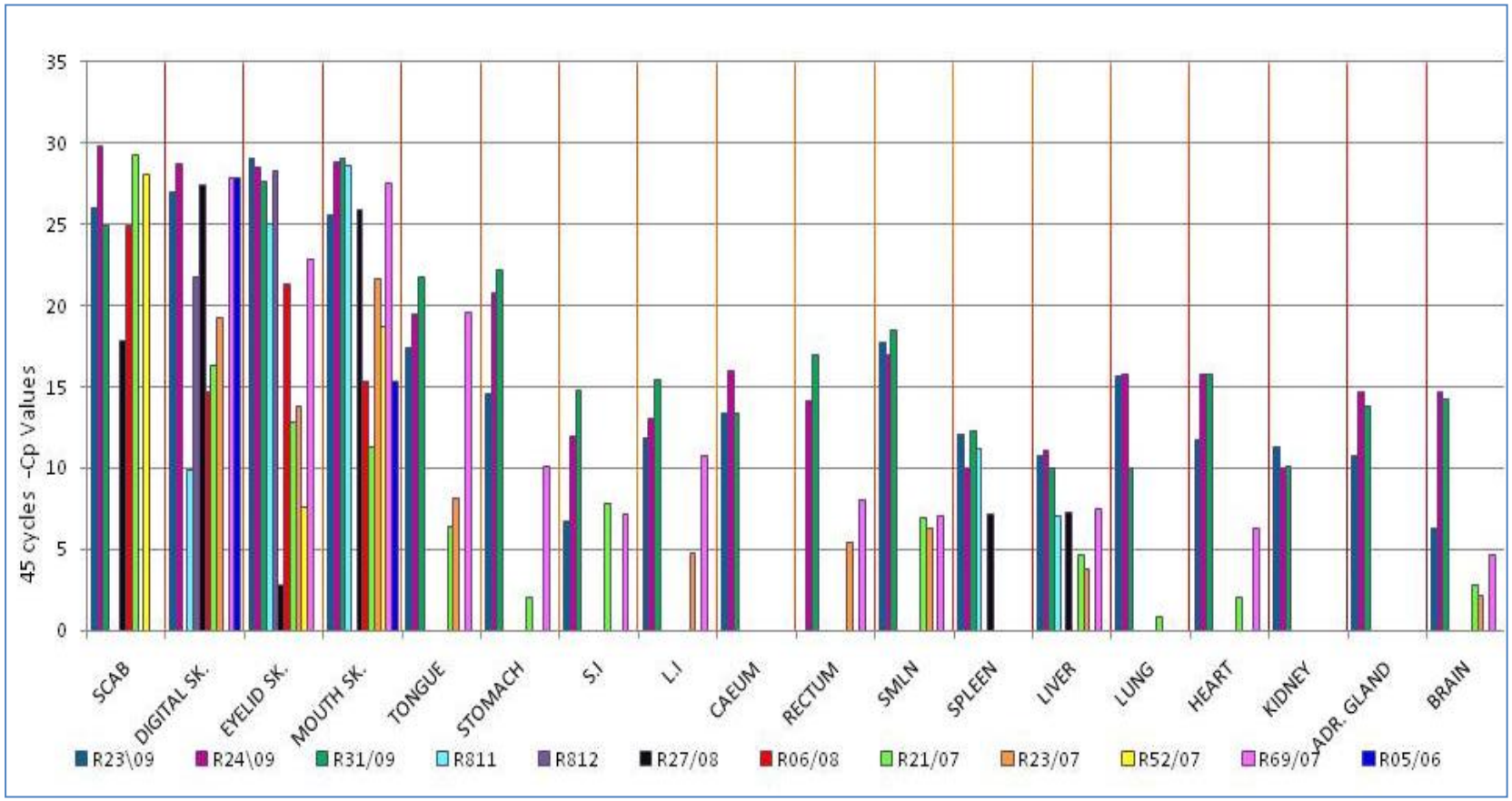


Figure 3.10 Graphic representation of qPCR results from the tissue samples of 12 SQPV infected red squirrels.
The magnitude of viral concentration in each sample is represented by an adjusted C_p score against the tissue sample. The adjusted Cp score was produced by subtracting the actual Cp score from 45 (the limit of the thermal cycles used for each reaction). Each sample was run in quadruplicate.

Table 3.9 contrasts the serology results from the 13 red squirrels (12 SQPV infected and one negative) with the qPCR results obtained from eyelid tissues. The findings clearly show that in SQPV infected red squirrels there is no correlation between serological status (as measured by the SQPV-specific ELISA) and the qPCR Cp values of positive samples (Pearson's correlation: r=-0.325, N=11, p=0.360 for digital skin; r=-0.074, N=13 p=0.828 for eyelid skin; r=0.006, N=12, p=0.986 for mouth skin and r=-0.746, N= 7, p=0.054 for scab). Whilst all six red squirrels with positive SQPV-ELISA results also have positive qPCR results, this is not true for the reverse. Six out of 13 red squirrels where both test results were available, tested negative in the ELISA but had positive qPCR results.

Table 3.9 Table comparing the serological status in SQPV-infected red squirrels with the mean qPCR Cp score of skin samples.

		Mean Cp score			
Animal	**ELISA OD_{450}**	**digital skin**	**eyelid skin**	**mouth skin**	**Scab**
R812/08	0.0	23.2	16.7	ns	ns
R31/09	0.0	ns	17.4	16.0	20.1
R811/08	0.0	35.1	20.0	16.4	ns
R06/08	0.0	30.3	23.7	29.7	20.1
R05/08	0.0	17.3	N	25.6	ns
R27/08	0.0	17.6	42.2	19.2	27.2
R86/07	0.0	N	N	N	ns
R69/07	0.21	17.2	22.2	17.5	ns
R23/07	0.26	25.8	26.3	23.4	ns
R21/07	1.38	28.7	31.2	33.7	15.7
R23/09	1.50	18.0	16.0	19.4	19
R52/07	1.60	ns	37.4	26.3	17
R24/09	2.96	16.3	16.5	16.2	15.2

Seven ELISA negative (OD_{450}: 0.0) and six ELISA positive red squirrels (OD_{450}: 0.21/2.96). Squirrels antibody status is compared with qPCR results (mean Cp score) taken from the most strongly positive results i.e. skin samples and scab. (N) qPCR negative result; (ns) not sampled.

3.3 Naturally-infected wild grey squirrels

Sainsbury and colleagues demonstrated that of the grey squirrels tested in England, the majority had antibody against SQPV. It appeared that disease in red squirrels was moving across the country following the spread of seropositive grey squirrels. Disease occurred in red squirrels just after the area was invaded by seropositive grey squirrels (Sainsbury & Gurnell 1995; Sainsbury *et al*., 1997; Sainsbury *et al*., 2000; Sainsbury 2008; Thomas *et al*., 2003). This pattern has also recently been documented in the red squirrel population in Scotland; in 2005 the first record of seropositive grey squirrels was documented and two years later the disease was found in red squirrels (McInnes *et al*., 2009).

To determine whether the qPCR assay could be used to detect SQPV in grey squirrels it was used on tissues taken from free ranging grey squirrels which were being controlled, by trapping and shooting, in the south of Scotland. Tests to detect viral DNA were carried out on both ELISA-positive and ELISA-negative grey squirrels.

3.3.1 qPCR analysis of grey squirrel tissue

Eight grey squirrels that had been trapped and shot during the grey squirrel control programme in the south of Scotland were obtained for qPCR analysis. The squirrels' carcasses were subjected to post-mortem examination as for the red squirrels and blood samples collected for testing in the SQPV ELISA. No gross abnormalities were found on post mortem examination. Different types of tissue samples were collected for subsequent SQPV DNA analysis and results for the I1L qPCR are presented in **Table 3.10**.

Table 3.10 Mean Cp value of qPCR analysis of grey squirrel tissues.

		SAMPLE									
Squirrel ID	**ELISA OD_{450}**	LUNG	SMLN	KIDNEY	LARGE INTESTINE	RECTUM	TONGUE	NASAL SKIN	EYELID SKIN	MOUTH SKIN	DIGITAL SKIN
530GR9NW	N	-	-	-	-	-	-	-	-	-	-
GR32HH	N	-	-	-	-	-	-	-	-	-	-
GR11HH	N	-	-	-	-	-	-	-	-	-	-
BG46GC	0.33	-	**+**	-	-	-	**+**	**+**	**+**	**+**	-
BG61LNF	1.6	-	-	-	-	-	-	-	**42.3**	-	-
BG62NW	0.73	-	-	-	-	-	-	-	**39.7**	-	**+**
BG54FF	1.84	-	-	-	-	-	-	-	-	-	-
BG57FD	2.32	-	-	-	-	-	-	-	-	-	-

(-) indicates the absence of detectable SQPV DNA; (+) indicates at least one positive replicates out of four; (N) negative ELISA result; SMLN: submandibular lymph node. The mean Cp value given represents the mean from the two independent assays (a total of eight out of eight positive results).

SQPV DNA was detected consistently only in the eyelid skin of two SQPV-ELISA positive squirrels (BG61LNF, BG62NW). In each of these samples all four replicates were positive in the qPCR reaction in two different assays. Digital skin samples from squirrel BG62NW were also qPCR positive as were submandibular lymph node (SMLN), nasal, mouth, eyelid skin and tongue samples from squirrel BG64GC. However, in all these instances only one replicate in four gave a positive result, with the other three replicates being negative.

Samples were therefore tested again using the same extracted DNA in order to confirm SQPV presence. For squirrel BG46GC this second assay was negative for SMLN, tongue and nasal skin as also the digital skin from BG62NW, whereas eyelid and mouth skin from BG64GC again gave one positive replicate out of four, while samples from the eyelid skin of the two seropositive squirrels (BG61LNF, BG62NW) were confirmed to be positive again with 4 out of 4 replicates giving an

overall positive result. A third assay was conducted again testing the DNA from squirrel BG64GC and the results were as is shown in **Table 3.11**.

Table 3.11 qPCR positive and negative results from squirrel BG64GC given in three different 1IL qPCR assays.

SMLN	Tongue	Mouth skin	Nasal skin	Eyelid skin
+	+	+	+	+
-	-	+	-	+
-	-	-	Not performed	-

(+) one positive replicate out of four; (-) negative results; SMLN: submandibular lymph node.

3.4 Discussion

The current dogma states that grey squirrels are the reservoir host for SQPV. Apart from one report in the literature of a grey squirrel showing clinical signs of disease and confirmed, by EM, to have poxvirus particles present in skin lesions (Duff *et al*., 1996), grey squirrels are generally considered to be unaffected by the virus. However, they are thought to be the source of the disease in red squirrels which, once infected, rarely survive in the wild (Sainsbury & Gurnell 1995; Sainsbury *et al*., 1997; Sainsbury *et al*., 2000; Sainsbury *et al*., 2008; Sainsbury 2008; Thomas *et al*., 2003; Tompkins *et al*., 2002). It is not known how the virus is transmitted from grey squirrels to red squirrels, particularly with the lack of clinical signs of infection in the grey squirrels, and some have speculated that transmission is more likely from red squirrels, with their overt signs of disease, to grey squirrels.

This chapter presents the refinement of a qPCR assay and its initial use to examine tissues taken from naturally-infected red and grey squirrels in an attempt to determine which tissues might be involved in the replication of the virus in these two species.

The post mortem findings confirmed that the only consistent lesions in red squirrels were found in the skin. As has been reported previously (Sainsbury & Ward 1996; Thomas *et al*., 2003; Tompkins *et al*., 2002), skin lesions were characterized by exudative erythematous dermatitis with scab formation. Often the lesions were localized to muco-cutaneus junctions extending onto the skin surface and severe necrotising lesions of mucosal surfaces were not frequently observed, with the exception of occasional lesions present on the tongue. Lesions were also present on other parts of the body, but the majority were presumed to be associated with puncture wounds or other areas of damaged skin.

It has been suggested that in red squirrels the infection may lead to a viraemia with a generalized spread of the virus (Atkin *et al*., 2010) but, in this thesis, there was no evidence of systemic disease in internal organs. In addition, several red squirrels were found with lesions only on one side of the body arguing against systemic spread of the virus. If viraemia had occurred lesions would be expected symmetrically in the target region of virus replication (e.g. periocular and digital skin).

Microscopically, skin lesions demonstrated hyperplasia and ballooning degeneration of the epidermis and the presence of pox-like intracytoplasmatic inclusion bodies. In contrast, none of the grey squirrels examined showed any macroscopic or microscopical signs of SQPV disease either externally or internally.

For the first time it was possible to follow post mortem examination of squirrel carcasses with an assay to determine which tissues were harbouring the virus and in which the virus might be replicating. Not surprisingly, the skin lesions from the red squirrels were found to contain most viral DNA (estimated from the standard curve to be in the region of eyelid, digital and mouth/nasal skin) and it is reasonable to predict that the skin in the red squirrel is the major site of virus replication. Indeed many poxviruses are known to replicate in the skin of the animals they infect. Viral DNA was also detected in all of the red squirrel tissues and organs examined suggesting systemic spread of the virus had occurred. However, it seems unlikely that the heart, brain, kidney and adrenal glands act as a site of replication given that viral DNA was not consistently detected in these tissues and the amount of DNA detected was also relatively low (estimated to be lower than 300 genome

copies/reaction). The SM LN and liver were consistently found to contain viral DNA and at a level higher than found in the heart, brain, kidney and adrenal glands. Whether or not this is an indication of the virus replicating in these tissues is, however, debateable. Some of the animals submitted for post mortem examination were already partially decomposed or damaged by scavengers, and so contamination of internal tissues with virus may have been a strong possibility. In addition, although great care was taken during the post mortem process, it is also likely that some cross-contamination with virus would have occurred. For example, in removing the brain it was necessary to cut through skin on the head that had clear poxvirus lesions. Nevertheless, this would not necessarily explain why the liver consistently appeared to contain more DNA than some of the other internal tissues (e.g. kidney). The viral DNA detected in the gastrointestinal tract was thought likely to be mainly derived from ingesting virus that was present around the mouth and head and that could also explain the viral DNA presence in a faecal sample from one red squirrel.

The tissue samples collected from the naturally-infected grey squirrels generally reflected those thought most likely to be positive for virus based on the results obtained from the red squirrel work. None of the eight grey squirrels examined were showing any external signs of pox disease and so there was no reason to believe that any were currently infected with the virus. Five, however, had tested seropositive in the SQPV ELISA and so were at least considered to have been exposed to the virus at some stage.

SQPV DNA was detected consistently in only two samples, the eyelid skin from two of the seropositve grey squirrels. The Cp score was extremely high (means of 39.7 and 42.3) for both samples raising the question as to whether or not they represented genuine positive results. Indeed the Cp scores were higher than those obtained reproducibly for the lowest dilution of virus DNA used in the standard curve (30 copies: Cp mean of 34.4 +/- 0.486) and higher than those obtained for the heart, brain, kidney and adrenal glands in the red squirrels, which had already been discounted as likely cross-contamination. However, since it was not known when the two squirrels in question might have been infected with the virus and because eyelid

skin in the red squirrel is certainly a target for virus replication, biologically it may be logical to treat these results as genuine positive results.

The analyses of grey squirrel tissues raised questions of how to interpret the results of the qPCR assays. Several samples, from grey squirrel BG46GC (**Table 3.10**) and the digital skin from squirrel BG62NW, each had one replicate out of four giving a positive Cp score in the qPCR assay. The Cp score in each case was very high (between 38 and 42.7), indicating that the amount of viral DNA in these particular replicates was very low if indeed viral DNA was present at all. De las Heras *et al*., (2005) reported that in a conventional PCR assay for Jaagsiekte sheep retrovirus (JSRV) not all replicates of a single sample need to give a positive result for the overall result to be considered positive. They argued that by only taking 500 ng of DNA per assay they were restricting the analysis to approximately 70,000 cells (assuming 7 pg of DNA per cell). If the virus was present at a frequency of less than one virus per 70,000 cells then they would not expect to obtain a positive result. Previous work by Holland *et al*., (1999) had proposed that the frequency of JSRV infection in ovine peripheral blood cells was approximately 1/240,000. The SQPV qPCR assay only used 200 ng of DNA as a starting point, representing approximately 28,000 cells, assuming the same calculation performed by De las Heras *et al*., (2005). However in this case if the single positive replicates do indeed represent genuine SQPV positive results it is difficult to know what biological significance this would carry.

For diagnosis of SQPV infection, clinical and post mortem findings, histopathology of lesions and electron microscopy are used commonly as well as serological tests; however, these tests are laborious, time consuming and not conclusive in all cases. The analysis of the red squirrel tissues by qPCR in this chapter also demonstrated the need for a confirmatory test for SQPV infection. Specifically one animal, R05/08, had initially been considered negative with respect to SQPV infection due to the lack of pathognomonic lesions. The qPCR test, however, demonstrated that viral DNA was present in the digital and mouth skin. It is assumed, given that the animal was also sero-negative for exposure to SQPV, that this animal was in the early stage of infection and lesions had not yet had time to develop. Thus, qPCR is able to detect subclinical infection or early stages of disease even when lesions are not clearly

visible. In contrast, a second squirrel, R86/07, was considered to be a case of squirrelpox based on external lesions, but no viral DNA was found in any of the tissues examined and no virus particles were detected in the skin lesions by EM. A study of red squirrels from the Isle of Wight and from Jersey, neither of which have a resident grey squirrel population, also reported the presence of squirrelpox-like lesions from which no virus could be detected (Simpson *et al*., 2010). These authors also suggested that pathological examination alone was not sufficient for accurate diagnosis of squirrelpox.

Some of the work described in this chapter was published (see **Appendix 2.0**) as:

McInnes, C.J., Coulter, L., Dagleish, M.P., **Fiegna, C**., Gilray, J., Willoughby, K., Cole, M., Milne, E., Meredith, A., Everest, D.J. & MacMaster, A.M. (2009). First cases of squirrelpox in red squirrels (*Sciurus vulgaris*) in Scotland. *The Veterinary Record,* **164**, 528-531.

LaRose, J.P., Meredith, A.L., Everest, D.J., **Fiegna, C**., McInnes, C.J., Shaw, D.J. & Milne, E. M. (2010). Epidemiological and postmortem findings in 262 red squirrels (*Sciurus vulgaris*) in Scotland, 2005 to 2009. *The Veterinary Record,* **167**, 297-302.

CHAPTER 4

EXPERIMENTAL INFECTION IN GREY SQUIRRELS

4.1 Introduction

The results presented in **Chapter 3** highlighted the difficulty in detecting SQPV DNA in naturally SQPV-infected seropositive grey squirrels. This may have been because these seropositive animals had been infected, but had already cleared the virus, long before they were taken for this study. As a result, tissues from experimentally infected grey squirrels were examined instead. This study was divided into two phases; firstly, tissues were examined from a previous experimental infection (Tompkins *et al*., 2002), performed to demonstrate that SQPV was responsible for the disease seen in red squirrels whilst leaving grey squirrels apparently unaffected. Secondly, a new experimental infection was performed using three different routes of inoculation and tissue samples were collected. This also served as a pilot study to determine the most appropriate route of infection, scarification (SCA) with topical application of the virus, intranasal (I/N) inoculation or subcutaneous (S/C) injection, for a subsequent time course experiment which will be discussed in detail in **Chapter 5**.

The objective of the first phase of the study was to determine whether or not virus DNA could be detected in tissues taken from animals in which the time of infection was defined. In the second phase, the time of infection was again defined, but many more tissues were examined. In addition, the routes of experimental inoculation were designed to simulate possible routes of SQPV infection in the field. The subcutaneous infection simulated insect-borne virus transmission, the intranasal route aerosol transmission and the skin scarification, already proved to be successful in grey and red squirrel experimental infections (Tompkins *et al*., 2002; Thomas *et al*., 2003), reproduced one of the commonest routes of infection for poxviruses in the wild, transmission through broken skin by direct contact or via fomites.

4.2 Analysis of tissues collected from experimentally infected grey squirrels

The experimental details are given in Tompkins *et al*., (2002). Briefly, 18 wild grey squirrels were trapped and housed in separate cages. Each cage was connected to another one through a horizontal tube in order to have contact between squirrels in the same group. Animals were divided into three groups and challenged by scarification: Group A (**Table 4.1**: IDs169/00 to 174/00) were infected with a tissue-culture adapted strain of SQPV (230 SPPV 1999); group B (**Table 4.1**: IDs 163/00 to 168/00) were infected with a pooled homogenate in phosphate buffered saline (PBS) of poxvirus skin lesions taken from six red squirrels found dead with squirrelpox in the UK between 1993 and 1996; and group C, (**Table 4.1**: IDs 157/00 to 162/00) were scarified with water to act as a mock-infected control. Animals in group A were challenged with virus at a titre of 1 x 10^7 $TCID_{50}$/ml. Animals in group B were challenged with virus at a concentration of approximately 1 x 10^5 SQPV particles per ml, as judged by electron microscopy (Tompkins *et al*., 2002).

Eight months later squirrels in groups A and B were re-challenged using the same inocula as before, with the animals being infected by both intranasal and intradermal routes as well as scarification. Each animal received; 0.1 ml of inoculum administrated in each nostril and 0.1 ml injected subcutaneously at the scapula as well as 0.1 ml applied topically to a fresh scar on the thigh. Animals were euthanized 41 days after the second challenge and tissue samples collected at necropsy.

4.2.1 qPCR analysis of tissues

Tissues had been collected from each animal at necropsy in 2001 and stored at -80°C until processed for qPCR analysis six years later. Skin from the site of scarification, salivary glands, spleen, kidney and liver samples were available for all 18 squirrels with the exception of squirrels 157/00 and 158/00 (mock-infected) where lung tissue was analyzed instead of spleen. DNA was prepared from each and subjected to qPCR analysis with I1L primers set.

All DNA samples from all tissues were negative with the exception of those prepared from the scarification sites of animals infected with the wild type virus. The results of the analysis are presented in **Table 4.1**.

The amount of virus DNA detected was relatively low as indicated by the high C_p values. Using the I1L primer set there was no viral DNA detected in Group A animals (**Table 4.1**). In group B (grey squirrels infected with wild type virus) virus DNA could be detected in skin from the site of scarification in all six animals. In five of these samples, all four replicates were positive, and in one animal (166/00), three out of four replicates were positive (**Table 4.1**).

Table 4.1 qPCR Cp values and standard deviation from analysis of scarified skin samples taken from 12 SQPV-challenged grey squirrels.

	Squirrel ID	I1L Detector Mean Cp value	Standard deviation
Group A	169/00	N	
	170/00	N	
	171/00	N	
	172/00	N	
	173/00	N	
	174/00	N	
Group B	163/00	39	±1.295
	164/00	38.3	±0.47
	165/00	36.6	±0.482
	166/00	37.9*	±0.597
	167/00	33.8	±0.189
	168/00	36.1	±0.627

Group A: six animals challenged with tissue culture adapted strain of SQPV. Group B: six animals challenged with pooled homogenate of SQPV from red squirrel skin lesions. Each sample was processed in quadruplicate and results are represented by the mean values. (*) three positive qPCR replicates out of four; (N) negative results.

The same results are summarized in **Table 4.2**, but giving the percent of positive results and the standard deviation range for each of the three groups utilized for the experiment. Group C (negative control) is also shown.

Table 4.2 qPCR analysis of 12 experimentally SQPV infected and six mock infected grey squirrels.

Squirrel Group	Mean group Cp % of positive	C_p SD range
A	N	/
B	37 100% positive	±0.18 ±1.29
C	N	/

Group A: six squirrels challenged with tissue culture adapted strain of SQPV. Group B: six squirrels challenged with pooled homogenate of SQPV from red squirrel skin lesions. Group C: Six negative control (mock-infected) squirrels. The middle column represents the mean C_p value obtained within each group. Group B: Percent of positive qPCR results with the relative standard deviation range (C_p SD range).

4.3 Pilot study on different routes of experimental infection of grey squirrels

4.3.1 Preparation of SQPV inocula and virus quantification

Preparation of SQPV inocula and subsequent infection of grey squirrels with wild-type virus, were prepared from scabs from a number of different diseased red squirrels (§**2.3.2**). The SQPV-ELISA was used to monitor whether or not the challenge had been successful. A second challenge followed six weeks after the first, using a mixed preparation containing scab material from the eyelid skin of a single SQPV infected red squirrel (squirrel ID: MP 08/0383) and virus grown in foetal lamb skin cells (FLSc) (§ **2.3.1.2**).

4.3.1.1 Wild-type virus inoculum (first challenge); qPCR quantification using the standard curve

A standard curve was prepared for the qPCR (as described in §**2.5.7.1**) to quantify the amount of SQPV DNA in the inoculum used for the first experimental challenge (**Figure 4.1**). SQPV DNA was extracted from 30 µl of the wild-type virus inoculum and eluted in 100 µl elution buffer (§**2.5.1.1**). Subsequently, 2 µl of the eluted DNA was quantified by qPCR. The results of the quantification are shown in **Table 4.3**, indicated as "SCAB MIX", and predicted the concentration of virus DNA to be 4.01

x 10^8 genomes/2 µl. Therefore, the virus preparation was estimated to contain approximately 1.7 x 10^{11} genome copies/ml. It was decided to use approximately 10^8 viral particles per ml for the experimental infection and consequently the virus inoculum was diluted 1:1000 in phosphate buffered saline (PBS) plus 5% v/v penicillin/streptomycin (100 units/ml and 0.1 g/l respectively) in order to obtain a final virus concentration of approximately 1.67 x 10^8 viruses/ml. This compares to the 10^5 virus particles/ml used in previous experimental infection studies (Tompkins *et al*., 2002).

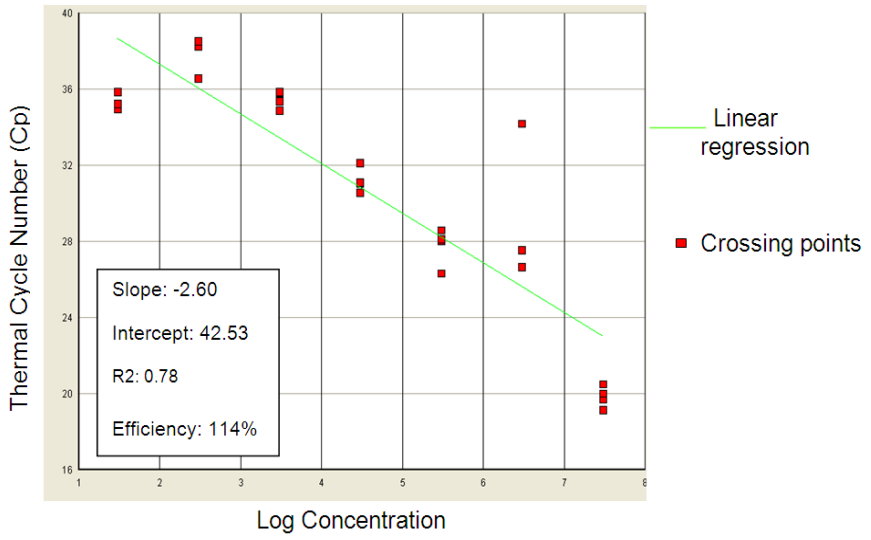


Figure 4.1 Standard curve obtained for quantification of the wild type virus inoculum used for the first challenge in the route of infection study.

Standard curve used to quantify the SQPV inoculums. Cp values are plotted against DNA concentration expressed in $\log_{10}$. The standard curve is obtained with seven fold serial dilutions (four replicates of each dilution, red square) of standard SQPV cosmid #86 containing the equivalent of 3 x 10^7 to 30 copies of the target gene. The relative coefficient of correlation (R2), slope, intercept and qPCR efficiency are indicated in the square inside the picture.

As shown in **Figure 4.1** the qPCR efficiency was greater than 100% (114%). Two factors could influence the slope value and consequently the qPCR efficiency; pipetting error between the standard dilutions of the SQPV cosmid samples used to construct the standard curve or the presence of a qPCR inhibitor in the standard.

Pipetting error is though the more likely explanation for this qPCR standard curve, as reflected in the high standard deviation (SD Cp) for several standard dilutions (**Table 4.3)**.

Table 4.3 Values obtained by qPCR using the relative standard curve method to quantify SQPV DNA extracted from the wild-type virus inoculum

Sample	Detector	Qty	Mean Cp value	SD Cp	Mean Qty	Std. Dev Qty
1.3	I1L	30000000	19.83	0.564		
1.3×10^{-1}	I1L	3000000	29.46	4.124		
1.3×10^{-2}	I1L	300000	27.76	0.995		
1.3×10^{-3}	I1L	30000	31.21	0.655		
1.4×10^{-4}	I1L	3000	35.39	0.391		
1.3×10^{-5}	I1L	300	37.97	0.93		
1.3×10^{-6}	I1L	30	35.32	0.377		
SCAB MIX	I1L	7.07×10^{8}	20.51	1.377	$\underline{4.01 \times 10^{8}}$	2.74×10^{8}

qPCR values derived from standard curve used to quantify virus load in SQPV scab mix (underlined value in the last line) used for the first experimental virus challenge. Each sample and dilution was assayed respectively in quadruplicate with seven fold SQPV cosmid #86 dilutions (from 1.3 to 1.30×10^{-6}; first column) and used to create the relative standard curve. (SCAB MIX): sample representing the SQPV inoculums quantification relative to two µl.

4.3.1.2 Mixed SQPV inoculum (2nd challenge)

All squirrels (with one exception) were re-infected with SQPV six weeks after the first virus challenge. For this second infection, SQPV was isolated from the eyelid scabs collected from a single red squirrel with confirmed squirrelpox disease and it was supplemented with SQPV grown in culture. To demonstrate the presence of SQPV in the scab preparation, DNA was extracted and analyzed with the conventional PCR assay. The SQPV DNA was detected using primers that amplify a product of 258 pb which corresponds to the I11L gene in the SQPV genome (McInnes *et al*., 2009). As shown in **Figure 4.2** SQPV DNA was detected in the eyelid scab preparation.

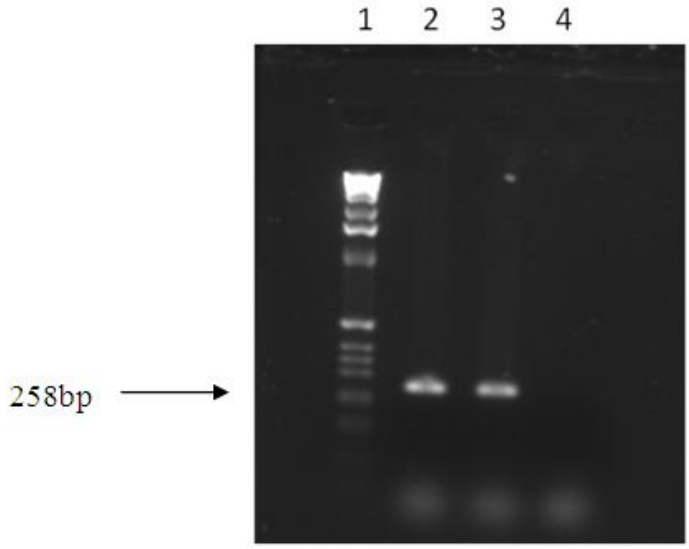


Figure 4.2 Agarose gel electrophoresis of the amplicon obtained from the red squirrel eye lesion.

Detection of SQPV DNA from an eyelid lesion of an infected red squirrel. Lanes: 1) Marker: Ready-loadTM 1Kb DNA ladder (Invitrogen); 2) Positive control; 3) Red squirrel eyelid lesion; 4) Negative control: water.

The eyelid scab preparation was supplemented with SQPV (Red Squirrels SPPV 230, FLM 19, 10 Feb 1999) grown in FLSc (as explained in §**2.3.1.2**). A series of $\log_{10}$ dilutions of concentrated cell culture grown SQPV were prepared and used in a titration assay to determine virus concentration. After seven days in culture the extent of the cytopathogenic effect (CPE) produced by each of the serial dilutions was assessed and the Log $TCID_{50}$/ml was calculated using the Spearman/Kärber method (Mahy & Kangro 1996). This indicated a virus concentration of 4×10^7 $TCID_{50}$ /ml. The final inoculum used for the second challenge, given six weeks after the first challenge, was prepared by mixing 2.5 ml of the single scab preparation with 0.1 ml of cell culture grown virus (**Table 4.4** indicated as SCAB & C.G.100 µL**)**. This ensured that there was a minimum concentration of (cell culture grown) viable virus of 1.5×10^6 $TCID_{50}$ /ml.

4.3.2 Experimental infection

Three routes of inoculation were investigated in order to evaluate the most appropriate route of infection in grey squirrels for subsequent experiments.

Twenty grey squirrels were divided into three experimental groups: A (N=7) for scarification (SCA), B (N=6) for intranasal infection (I/N) and C (N=7) for subcutaneous infection (S/C) (**Table 4.4**). Males and females were equally distributed between groups. The remaining animal (the twenty-first), squirrel D, displayed a severe respiratory depression after induction of anaesthesia and it was decided not include it in any experimental group.

Table 4.4 Summary of squirrel groups and their ID, route of infection, gender and SQPV inocula type and quantity used for the 1st and the 2nd challenge.

Group	Squirrel ID	Route of infection	Gender	First challenge Inoculum (type and quantity)	Second challenge Inoculum (type and quantity)
GROUP A	B	SCA	M	SCAB MIX 100µL	SCAB & C.G.100µL
	H	SCA	F	SCAB MIX 100µL	SCAB & C.G.100µL
	K	SCA	F	SCAB MIX 100µL	SCAB & C.G.100µL
	O	SCA	M	SCAB MIX 100µL	SCAB & C.G.100µL
	Q	SCA	M	SCAB MIX 100µL	SCAB & C.G.100µL
	S	SCA	F	SCAB MIX 100µL	SCAB & C.G.100µL
	W	SCA	M	SCAB MIX 100µL	SCAB & C.G.100µL
GROUP B	F	I/N	M	SCAB MIX 100µL	SCAB & C.G.100µL
	L	I/N	M	SCAB MIX 100µL	SCAB & C.G.100µL
	M	I/N	F	SCAB MIX 100µL	SCAB & C.G.100µL
	P	I/N	M	SCAB MIX 100µL	SCAB & C.G.100µL
	T	I/N	F	SCAB MIX 100µL	SCAB & C.G.100µL
	V	I/N	M	SCAB MIX 100µL	SCAB & C.G.100µL
GROUP C	A	S/C	F	SCAB MIX 100µL	SCAB & C.G.100µL
	E	S/C	M	SCAB MIX 100µL	SCAB & C.G.100µL
	G	S/C	M	SCAB MIX 100µL	SCAB & C.G.100µL
	I	S/C	F	SCAB MIX 100µL	SCAB & C.G.100µL
	J	S/C	F	SCAB MIX 100µL	SCAB & C.G.100µL
	N	S/C	M	SCAB MIX 100µL	SCAB & C.G.100µL
	R	S/C	M	SCAB MIX 100µL	SCAB & C.G.100µL

SCA: scarification; I/N: intranasal; S/C: subcutaneous; M: male; F: female; SCAB MIX: 100 µl of virus inoculum prepared from a mixture of scabs from SQPV infected red squirrels. SCAB & C.G (Cell Grown): 100 µl of inoculum prepared from SCAB (single scab preparation) and SQPV adapted to cell culture.

4.3.2.1 Clinical score

During the course of the experiment, animals were examined daily for skin lesions and other clinical signs of disease. Animals were given a daily clinical score (based on the **Table 2.9** §**2.6.4**) and their body weight was recorded at least every three days throughout the experiment. The Home Office licence stipulated that animals reaching a score of eight or more on three consecutive occasions would be euthanized. None of the SQPV challenged grey squirrels required to be removed prior to the end of the experiment (eight weeks post first challenge). **Figure 4.3** gives an example of a daily clinical score for the 21 grey squirrels based on the clinical scoring system used to assess the SQPV infected grey squirrels.

Date: 8 April 2008

Squirrel ID	Pox lesion	Distal lesion	Appetite/ depression	Faeces/ urine	Weight (g)	Weight loss score	TOTAL SCORE
A			2A		530	2	4
B			2A		610		2
D (not infected)					561		
E			2A		593		2
F					645		
G			2A		466		2
H					485		
I			2A		508		2
J					536.5		
K					595	2	2
L			2A		654		2
M			2A		606		2
N					542		
O					609.5		
P					600		
Q			3A		522		3
R			2A		486		2
S			2A		503		2
T			2A		514		2
V			2A		596		2
W					734		

Notes:

Figure 4.3 Example of daily clinical score at six weeks post infection.

Example clinical score sheet from 8th April 2008 indicating the daily assessment of the experimentally infected grey squirrels. Scores may range from a minimum of 0 (blank) to a maximum of 8. (A) appetite.

None of the grey squirrels in any of the three inoculation groups showed any detectable signs of illness or secondary skin lesions and the survival rate was 100% in each group. Food and water intake was the only parameter which scored due to a reduction of animal appetite. The animals were closely monitored and although the scores gave rise to concern the animals appeared to be in good health throughout the eight weeks observation period. The maximum clinical score recorded was six for animal T, but again the animal did not appear to be suffering from disease or to be in ill-health. The overall general clinical score fluctuated throughout the eight weeks period, principally as a result of the reduced consumption of food and concomitant weight loss, but it was considered that this was probably caused by the stress of wild squirrels being kept in captivity.

On day 0, all 21 grey squirrels appeared clinically normal (squirrel D was included throughout the period of the experimental infection and served as a non-infected control). On day 0 the body weight of 21 squirrels ranged between 493g to 714g. The physical condition and the body weight of all 21 grey squirrels remained relatively stable without any dramatic variation during and after the two challenges with virus. Mean body weight of each group of virus challenge throughout the course of the experiment (**Figure 4.4**) showed that at no time did any squirrel lose more than 20% (maximum individual body weight lost for squirrel T at eight weeks PI was -18.7%) of their starting weight when first captured from the wild. At no point did the clinical score reach eight for any of the animals. No clinical signs of SQPV infection (e.g. **Figure 4.5**) were observed at any time in any of the three challenged groups (A, B, C) and at no point did any of the squirrels score for the presence of external skin lesions.

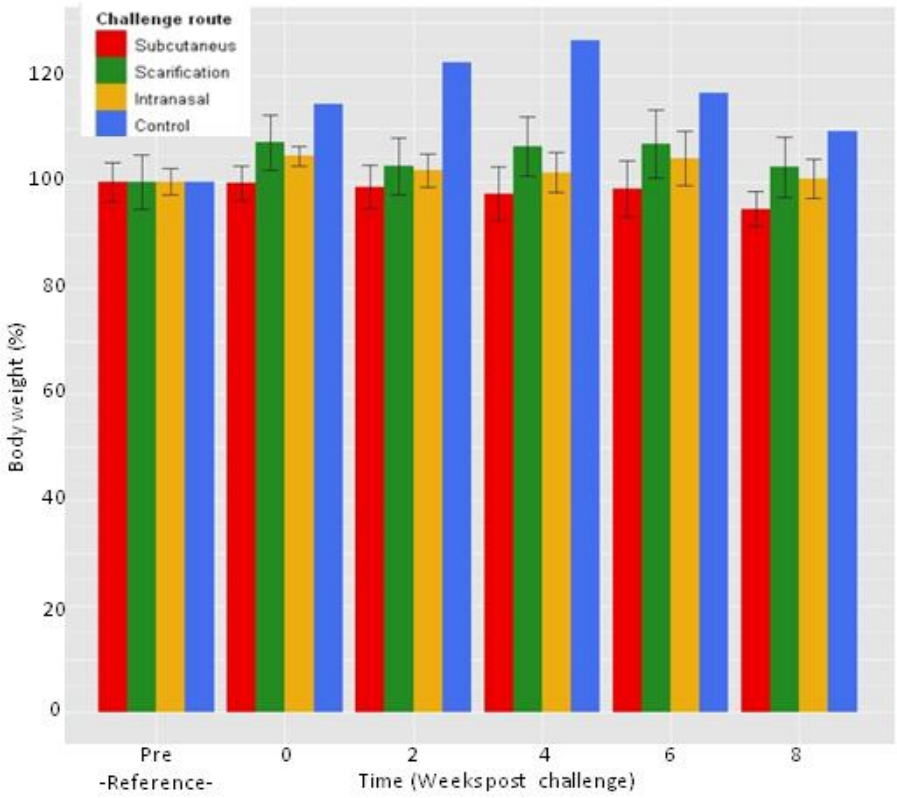


Figure 4.4 Comparison of body weight between the three groups of grey squirrels infected with SQPV via different routes.

Change in the body weight (%) during the experiment recorded at two-week intervals for animal groups challenged by scarification (green), subcutaneous (red) and intranasal (yellow) inoculation, compared to the pre-challenge values (pre-Reference-) expressed as 100%. Error bars represent 95% Confidence Intervals. The uninfected squirrel (D) is indicated in blue.

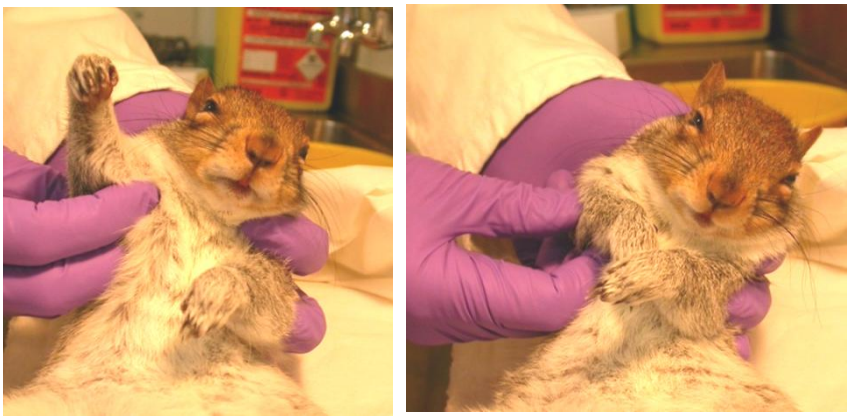


Figure 4.5 Healthy SQPV infected grey squirrel, six weeks post infection.

Typical signs of SQPV disease include erythematous dermatitis with lesions around the eyelids, mouth, nose and digital skin. Experimentally infected grey squirrels did not show any clinical signs of disease.

4.3.2.2 Serology

The squirrels were tested for serological evidence of previous SQPV infection prior to their inclusion in the experiment and all were found to be negative. Thereafter blood samples were collected at two-weekly intervals throughout the course of the study and the SQPV-antibody response measured by ELISA (Sainsbury *et al*., 2000). For more details see §**2.4**. The individual results for each route of infection are presented in **Figure 4.6**. Previously, Sainsbury *et al*., (2000) indicated that the point at which ELISA results positive for SQPV antibody detection can be differentiated from negative results should be set at an optical density (OD_{450}) of 0.20 at 450 nm. The overall serological response of the 20 grey squirrels challenged with SQPV was not as high as anticipated and none of the three routes of infection (scarification, intranasal and subcutaneous) elicited a strong antibody response either after the first or the second challenge. Results from all three groups stayed below the cut off point of 0.2 OD_{450} units with just the exception of one squirrel which was intranasally challenged (squirrel V). The fact that the antibody levels did not rise above the 0.2 OD_{450} cut off point did raise the question as to whether or not any of the infections had been successful and indeed whether or not the virus inoculum had been viable.

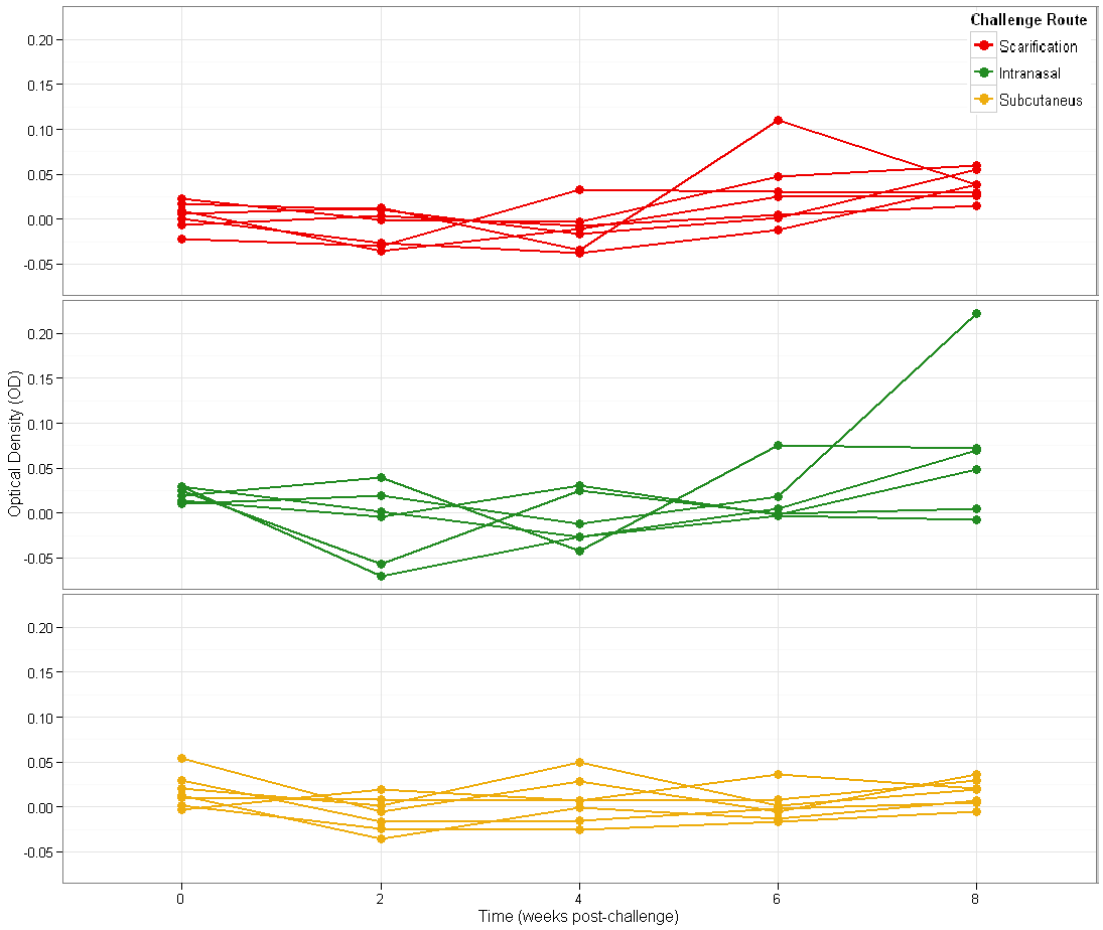


Figure 4.6 Individual optical density (OD) values for a direct ELISA testing for the presence of SQPV antibodies among eight weeks.

Time course of the serological response against SQPV recorded by direct ELISA for blood samples collected at two-week intervals. Squirrels belonging to the three groups were challenged by scarification (redlines), intranasally (green lines) and subcutaneous routes (yellow lines). At week six animals were re-challenged with SQPV by the same route of infection used for the first challenge for each group.

4.3.2.3 Post Mortem Examination

The 21 squirrels were sacrificed eight weeks after the first infection (two weeks after the second challenge). **Figure 4.7** shows animals E (subcutaneous), K (scarified) and W (scarified) illustrating examples of typical body condition from two out of three different routes of infection. Squirrels from the intranasal and subcutaneous inoculation groups did not show any visible lesions (e.g. squirrel E) whereas animals K and W showed scarified skin at different stages of healing and the closer view of

animal K (**Figure 4.7** bottom photo) exhibits a very mild erythematous dermatitis, but without any evident scab formation.

Figure 4.7 Examples of grey squirrels demonstrating healthy body condition.

From left to the right: squirrel E: subcutaneous challenge. Squirrels K and W: challenged by scarification. Note the differences in healing and fur growth in squirrel K compared to W. Both were scarified at the same time and sacrificed at the same day. Below is a closer view of scarified skin area from squirrel K.

Tissue samples (**Table 4.5**) were collected at post mortem examination for qPCR analysis and histopathology.

Table 4.5 Tissue samples collected during post mortem examination from all infected grey squirrels.

Lymphoreticular System	Integumentary system	Gastrointestinal system	Respiratory system	Endocrine gland	Urogenital system
SMLN Palatine tonsil Spleen Other lymph node*	Digital skin Nasal skin Scarification site Axillary skin Perianal scent gland* Mammary gland* Prepuce skin*	Parotid gland Tongue	Lung	Adrenal gland	Kidney

*Additional samples from some squirrels

The gross pathological examination was performed following the protocols shown in §**2.7**. No gross lesions obviously associated with a viral infection were observed in the 20 challenged grey squirrels. All examinations failed to reveal gross pathology in any visceral organs or skin tissue other then the scarified skin area. Two squirrels (F, M) did exhibit enlarged submandibular lymph nodes although the cause of this was not known (see also **Table 4.6-B-**).

4.3.2.4 Histopathology

Histopathological examination was carried out on tissue samples from the three inoculated groups. The samples were fixed in 10% formal saline, embedded in paraffin wax and stained with haematoxylin and eosin (H&E) staining. The samples were examined for abnormalities indicative of poxviral infection. **Table 4.6** (**-A-**, **-B-** and **-C-**) provides summaries of the histological findings.

The main feature of poxvirus infection is the presence of intracytoplasmic eosinophilic inclusion bodies thought to represent replicating virus within the cells (Himsworth *et al*., 2009; Schmiedeknecht *et al*., 2010). However none were present in any of the tissues examined. Other features may include ballooning degeneration, marked hyperplasia, acantholysis and epidermal spongiosis. These histological changes were observed more consistently in group A within the scarified skin samples (**Figure 4.8-A-** and **–B-**). The histopathological features of these lesions

were characterized by dermatitis with epidermal changes which varied their relative proportion between the individual samples. This included moderate epidermal hyperplasia and the presence of focal spongiotic changes with mild intracellular oedema in the epidermis, mild to moderate acantholysis of the keratinocytes with sporadic dyskeratotic keratinocytes with pyknotic nuclei (e.g. **Figure 4.8-A-** slide **C**, **Figure 4.8-B-** slide **E**). A mixed inflammatory cell infiltrate (mainly lymphocytes, neutrophils and macrophages) was observed in all seven samples of scarified skin, mainly in the superficial and mid dermis, and in one case it extended into the panniculus. Exocytosis was not a marked feature but was recognized in three samples (animals Q, H and K) mainly due to a lymhocytic infiltrate. The presence of sero-cellular crusts on the surface of the epidermis was observed in two samples (**Fig. 4.8-A-** slide **A** and **Figure 4.8-B-** slide **D**) and in only one case the histopathological examination revealed an area of dermal ulceration (**Figure 4.8-A-** slide **D**). In contrast, the histological results of the scarified skin from animal W only showed a very mild inflammatory infiltrate in the superficial dermis with no evident epidermal hyperplasia (**Figure 4.8-A-** slide **F**).

Other tissue types were also examined as indicated in **Table 4.6.** However, there were no pathological changes indicative of pox viral infection. **Figure 4.8-C-** slides **G** to **I**, show examples of nasal and eyelid skin from one intranasally infected animal (squirrel L) and one squirrel (N) subcutaneously infected. However, none of these samples had histopathological changes that could easily be attributable to SQPV infection.

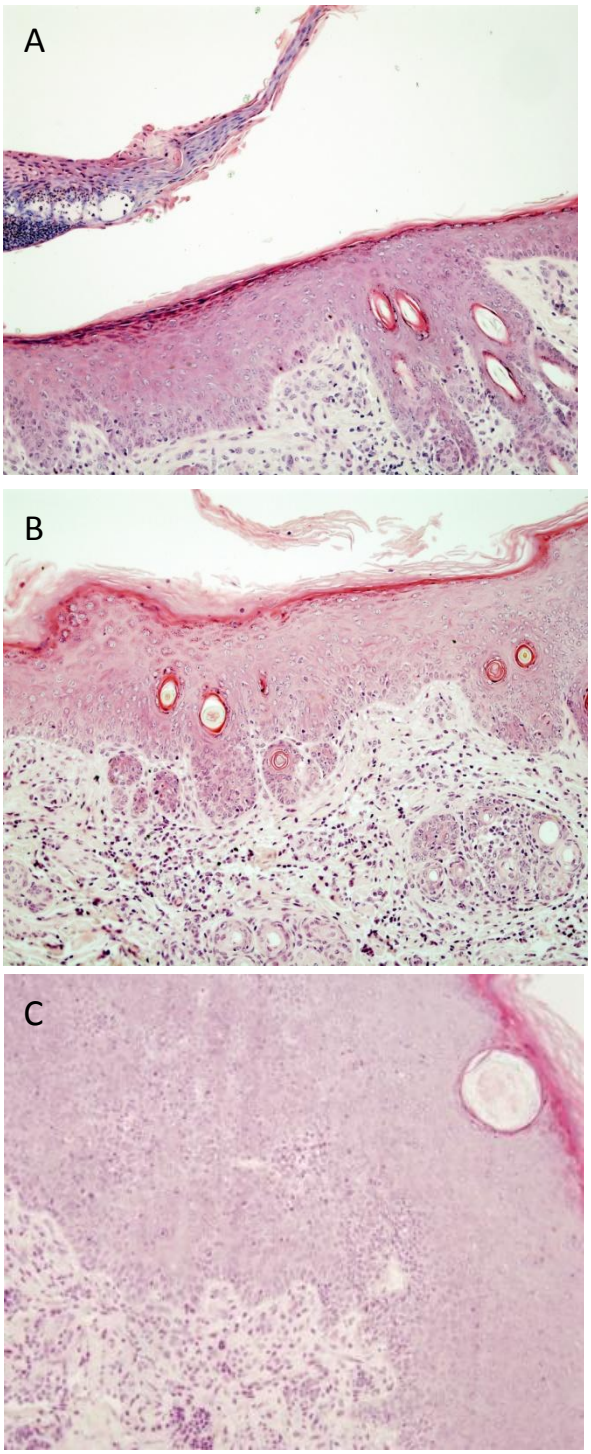


Figure 4.8-A- Histopathological changes 14 days after second SQPV challenge using the scarification route of inoculation. **Haematoxylin and Eosin (H&E) stain.**

(A) Squirrel B: SQPV scarified skin. Moderate lymphocytic/macrophagic dermatitis, moderate hyperplasia, presence of mild spongiosis with occasional acantholytic cells. Sero-cellular crust with degenerate neutrophils. Original magnification (OM) x 100. **(B)** Squirrel Q: SQPV scarified skin. Moderate hyperplasia, mild spongiosis and acantholysis at the dermal-epidermal junction. Mild lymphocytic exocytosis with marked dermatitis in the superficial and mid dermis. OM x 100. **(C)** Squirrel K: SQPV scarified skin. Moderate hyperplasia, presence of spongiosis. Necrotic cell debris in the epidermis and foci of acantholysis. Mild lymphocytic exocytosis. OM x 100.

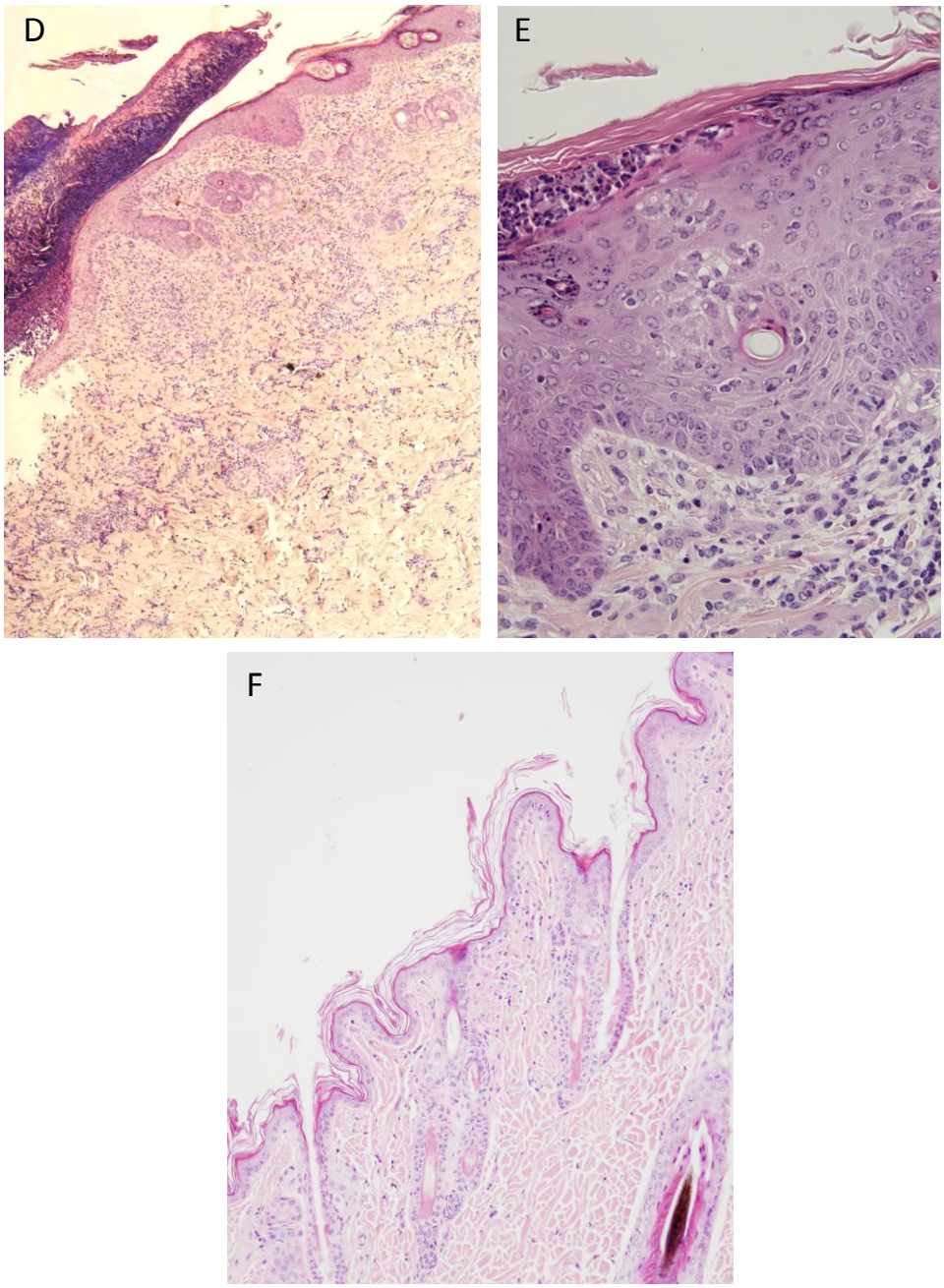


Figure 4.8-B- Histopathological changes 14 days after second SQPV challenge using the scarification route of inoculation. Haematoxylin and Eosin (H&E) stain.

(D) Squirrel H: SQPV scarified skin. Area of ulceration and serocellular crusting with many degenerate neutrophils infiltrating the underlying dermis. Mild to moderate spongiosis. Marked mixed inflammatory infiltrate extending to the lower panniculus. OM x 100. **(E)** Squirrel H: SQPV scarified skin. Presence of degenerate neutrophils in the upper epidermal and corneal junction. Moderate spongiosis and foci of mild acantholysis. Presence of a few inflammatory cells infiltrate in the epidermis and sporadic dyskeratotic keratinocytes. Marked inflammatory infiltrate in the lower dermis. OM x 200. **(F)** Squirrel W: SQPV scarified skin. Normal appearance of the skin. Very mild inflammatory infiltrate in the upper dermis. OM x 100.

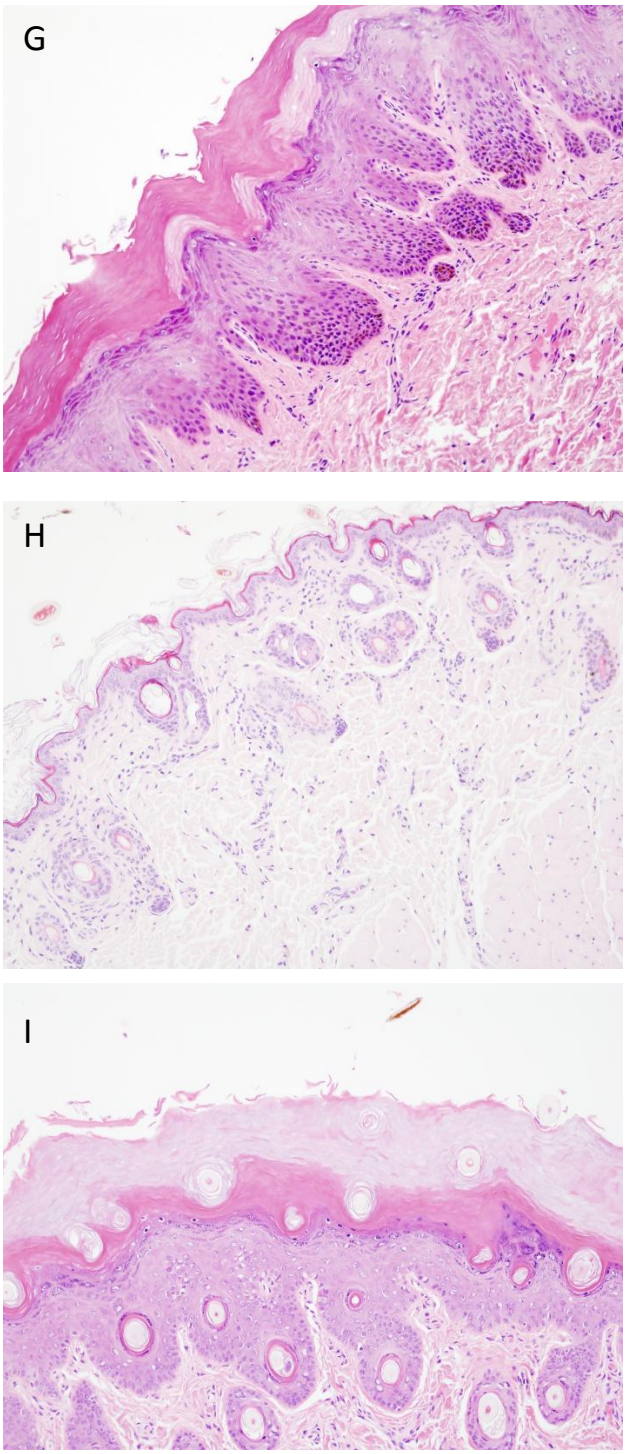


Figure 4.8-C- Skin histopathology 14 days after second SQPV challenge following the intranasal route of infection and subcutaneous. Haematoxylin and Eosin (H&E) stains.

(G) Squirrel L: nasal skin. Intranasal route of infection. Moderate spongiosis. Absence of inflammatory infiltrate in the lower derma. Original magnification x 100. **(H)** Squirrel L: eyelid skin. Intranasal route of infection. Normal aspect of the eyelid skin. Mild lymphocytes infiltrate in the upper derma. Original magnification x 100. **(I)** Squirrel N: nasal skin. Subcutaneous route of infection. Mild spongiosis and presence of intracellular oedema in the epidermis. Absence of inflammatory infiltrate. Original magnification x 100.

Table 4.6-A- Histopathological examination of samples collected post mortem from group A. challenged by scarification.

	Squirrel ID						
	B	**H**	**K**	**O**	**Q**	**S**	**W**
Digital skin	/	/	/	/	/	(*)SP	/
Nasal skin	/	(+)LyInf in SupD and DeepD	(*)SP	(*) and (**) SP; (+) AC	(*)SP	/	(*)SP
Eyelid skin	/	/	(**)SP	(*)LyInf in SupD	/	(*)LyInf in SupD	/
Scarified skin	SP(*); AC(+); Scab; (***)Ly/MaInf up to Pan.	(**)SP; U; Scab; (***) MixInf from SupD to Pan; (++)NeDe underlying dermis	(*)SP, AC; NCell in one hair follicle; (*)NeInf in SupE; (**)Ly/MacInf in SupD and DeepD	(**) SP; (+) AC, (**)Ly/MacInf in SupD and DeepD with periadnexal orientation	(**) SP; (**)Ac; (**)D; with Ly/MacInf in SupD and DeepD	(**)SP; (*)Ac; (**)Ly/Mac Inf in supD	(**)SP, (+)AC
Axillary skin	/	/	/	/		/	/
Adrenal gland	/	/	/	/	/	/	/
SM LN	/	/	/	/	/	Appears depleted	/
Scent gland	Ns	Ns	Ns	Ns	/	Ns	/
Parotid	/	/	/	/	/	/	/
Kidney	/	(^{0})NLy in cortex	/	/	/	/	(*)Nephr itis; (++)LyInf (+) NeInf
Spleen	/	/	/	/	/	(++)NeDeg in sinus	/
Lung	/	(*)ChInfl	/	/	/	/	/
Palatine tonsil	/	Ns	/	/	/	/	/
Tongue	/	/	/	/	(+)N	/	/

SP: Spongiosis; **AC**: Acantholysis; **LyInf**: Lymphocytic infiltrate; **NeInf**: Neutrophil infiltrate; **MaInf**: Macrophage infiltrate; **Ly/MaInf**: Infiltrate of lymphocytes/macrophages; **MxInf**: Mixed inflammatory infiltrate; **NeDeg**: Degenerate neutrophil; **U**: Ulceration; **N**: necrosis; **NCell:** Necrotic cell/debris; **NLy:** Foci of necrotic lymphocytes; **LyFoci**: Lymphoid foci; **ChInfl**: Cronic inflammation; **Scab:** Sero-cellular crust on epidermis; **SupE**: superficial in epidermis; **SupD**: Superficial dermis; **DeepD**: medium to deep dermis; **Pan**: Profundus extending to panniculus; (*****) Mild (******) Moderate (*******) Marked; (**+)** occasional (++) several; (**Ns)** no sample; (/) normal.

SM LN: submandibular lymph node;

Table 4.6-B- Histopathological examination of samples collected post mortem from group B. Intranasal challenge.

	Squirrel ID					
	F	**L**	**M**	**P**	**T**	**V**
Digital skin	/	/	/	/	(*)SP	/
Nasal skin	/	(**)SP	(*)SP	(*)SP	/	(**)SP
Eyelid skin	/	(*)LyInf in SuoD	/	(*)SP	/	(*)LyInf in SupD
Axillary skin	/	(++)U with NeInf	/	/	/	/
Adrenal gland	/	/	/	/	/	/
SM LN	reactive	/	reactive	/	/	Ns
Scent gland	Ns	/	Ns	/	Ns	/
Parotid	/	(+)NeInf with loss of acini	/	/	/	/
Kidney	/	/	/	/	/	(+)Pro, depressed area in cortex
Spleen	/	/	/	/	/	/
Lung	/	/	/	/	/	/
Palatine tonsil	/	Ns	Ns	/	/	/
Tongue	/	/	/	/	/	/

SP: Spongiosis; **LyInf**: Lymphocytic infiltrate; **NeInf**: Neutrophil infiltrate; **Pro**: Proteins in a few tubules; **U**: Ulceration; **N**: necrosis; **NCell:** Necrotic cell/debris; **NLy:** Foci of necrotic lymphocytes; **LyFoci**: Lymphoid foci; **SupD**: Superficial dermis; (*****) Mild (******) Moderate; (**+)** occasional (++) several; (**Ns**) no sample; (/) normal.

SM LN: submandibular lymph node;

Table 4.6-C- Histopathological examination of samples collected post mortem from group C. Subcutaneously challenged.

	Squirrel ID						
	A	**E**	**G**	**I**	**J**	**N**	**R**
Digital skin	(*)SP	/	/	/	/	/	/
Nasal skin	(*)SP	(*)SP	(*)SP	(**) SP	(*)SP	(*)SP; (*)Ac	(*)SP
Eyelid skin	very(+)LyInf in SupD	(*)SP	(*)SP; (*) LyInf in SupD	/	/	/	/
Axillary skin	/	/	/	/	/	/	/
Adrenal gland	/	/	/	/	/	/	/
SM LN	/	(+) NLy	/	/	/	/	Ns
Scent gland	Ns	Ns	Ns	Ns	/	Ns	/
Parotid salivary gland	/	Ns	/	/	/	/	/
Kidney	/	/	/	/	/	/	/
Spleen	/	(++)NeDeg in sinus	/	/	/	/	/
Lung	/	(+) interstitial lymphocytes and parasite	/	/	/	/	/
Palatine Tonsil	/	Ns	Ns	/	Ns	Ns	Ns
Tongue	/	/	/	/	/	/	/

SP: Spongiosis; **LyInf**: Lymphocytic infiltrate; **NLy:** Foci of necrotic lymphocytes; **NeDeg**: Degenerate neutrophil; **NLy:** Foci of necrotic lymphocytes; **SupD**: Superficial dermis; (*) Mild; (**) Moderate; (+) occasional; (++) several. (**Ns**) no sample; (/) normal.

SM LN: submandibular lymph node;

4.3.2.5 qPCR detection of SQPV DNA in selected tissues from squirrels inoculated by different routes

Results from the examination of naturally-infected red squirrels (**Chapter 3**) and the histological findings already presented in this chapter suggested that the main focus of the qPCR analysis should be the skin and other tissue types that either showed macroscopic lesions, histological abnormalities, or the presence of viral DNA (qPCR positive results). In addition to skin samples (eyelid, nasal, digital, axillary, scarified skin) samples from the lymphatic, respiratory and other integumetary (perianal scent gland as indicated in the table below†) organs were analysed (**Table 4.7**).

Table 4.7 qPCR results for tissues samples from grey squirrels challenged by three different routes of inoculation.

				Tissues								
Sq. ID	Route Inoc.	SM LN	Other lymph	Eyelid skin	digit. skin	Nasal skin	Scar skin	axill. skin	tongue	lung	scent gland	spleen
B	SCA	N		N	37.5**		39.2	39.2**				
H	SCA	N		37.2	N	N	36.9	N				
K	SCA	37.8		N	38.7**	N	37.4	39.5				
O	SCA	N		N	N	N	41.8	N			N	N
Q	SCA	N		N			36.8	N	37.3*		N	
S	SCA	N		N	37.5*		30.8	43.1**			39.9	39.3***
W	SCA	N		38.4***	34.5		N	N			41.4**	41.4*
F	I/N	N	N	N		N		N		N		
L	I/N	N		37.9***	N	40.1		N		N	N	
M	I/N	N	N	N	N	N		N		43.1**	N	
P	I/N	N		N	N	39.7**		N		N		
T	I/N	N		N		N		N		N	N	
V	I/N	N		37.4***		39.7***		N		N	37.8**	N
A	S/C				N	N						
E	S/C			N	N	N		N				N
G	S/C			N		N		N				
I	S/C					N		N				
J	S/C					N		N				
N	S/C					N		N				
R	S/C					N						

(N) negative results; Other lymph: Other lymph nodes (brachial lymph node); Digit. skin: digital skin; axill. skin: axillary skin; Scar. skin: scarification skin site. Scarification (SCA); intranasal route (I/N) and subcutaneously (S/C) route. (*) One positive qPCR replicate out of four. (**) Two positive qPCR replicates out of four. (***) Three positive qPCR replicates out of four.

As expected, the results from scarified skin samples in group A were positive (six out of seven squirrels). Within this group, the majority of other positive results were associated with the integumentary system (e.g. eyelid, digital and axillary skin as in **Table 4.7**). There was also one positive result out of the four replicates for tongue (squirrel Q). In addition, the spleens from two squirrels (S and W) were positive but with a higher mean Cp (mean 40.4) compared to the scarified skin (Cp mean 37.2).

In group B (I/N) positive results were again mainly associated with skin, 3 nasal skin samples positive out of six, but of more interest, there were also two positive eyelid skin samples; and in the case of squirrel V there was, in addition, a positive result in the scent gland (**Table 4.7**). Unexpectedly, the mean Cp value of eyelid skin was lower (37.7) than the Cp mean of nasal skin (39.8). All samples analyzed from group C (S/C) were negative.

The qPCR results were compared with the histopathological findings in order to correlate the confirmed presence of viral DNA with any pathological change (**Table 4.8**). Tissues at the site of inoculation were, understandably, associated with positive qPCR results. In the case of group B, the detection of viral DNA in the lung tissue of squirrel M is thought unlikely to be the result of viral replication given the low virus concentration (mean Cp value 41 with only 2 out of 4 positive replicates). The positive results for the eyelid skin in squirrels V and L may be due to the virus reaching the eyelid through the nasal cavity via the nasolacrimal duct or due to grooming behaviour of the animal. Significantly, Cp values from the eyelids were lower (larger amount of virus) compared to the actual site of inoculation, suggesting the possibility of viral replication and possible tropism for eyelid skin. This was also true for group A where squirrels S and W had positive results for eyelid skin but without any obvious pathological changes. The qPCR positive results for tongue and spleen from squirrels Q and S respectively do not appear to be significant because of the low concentration of viral DNA found which was consistent with minor histopathological changes (see **Table 4.6-A-)**.

Table 4.8 Histopathological abnormalities versus qPCR results.

		DIGIT	NASAL	EYELID	SM LN	LUNG	TONG.	SPLEEN	AXILL.	SCARIF	SCENT GLAN.
SCARIFICATION	B	37.5**		N	N				39.2**	39.2	
	H	N	N	37.2	N				N	36.9	
	K	38.7**	N	N	37.8				39.5	37.3	
	O	N	N	N	N				N	41.8	N
	Q			N	N		37.3*		N	36.8	N
	S	37.5*		N	N			39.3**	43.1**	30.7	39.9
	W	34.5		38.4***	N				N	N	41.4**
INTRANASAL	F		N	N	N	N			N		
	L	N	40.1	37.9***	N	N			N		N
	M	N	N	N	N	43.1**			N		N
	P	N	39.7**	N	N	N			N		
	T		N	N	N	N			N		N
	V		39.7***	37.4***	N	N			N		37.8**
SUBCUTANEUS	A	N	N								
	E	N	N	N					N		
	G		N	N					N		
	I		N						N		
	J		N						N		
	N		N						N		
	R		N								

First row: Digit: digital skin; SM LN: submandibular lymph node; Tong: tongue; Axill: axillary skin; Scarif: skin scarified with SQPV; Scent Glan; perianal scent glands.

(N) negative qPCR results; (*) one qPCR positive replicate in four; (**) two qPCR positive replicates in four; (***) three qPCR positive replicates in four. Blank space: samples not analized.

Highlight in yellow: histological changes; in blue: qPCR positive results; in green: both histopathological changes and qPCR positive results.

4.4 Discussion

The results of this preliminary experiment influenced the design of the time course study (**Chapter 5**) in a number of important ways. The qPCR results from wild caught grey squirrels **(Chapter 3**), and the experimental infection study (Tompkins *et al*., 2002) as well as the grey squirrel infection study described here, were all broadly similar and confirmed the difficulty of establishing and detecting a SQPV infection in grey squirrels.

qPCR analysis of grey squirrels used in the Tompkins study indicated that the wild-type virus inoculum may have been more adapted to grey squirrels than the tissue culture grown virus. This was because it elicited the greatest antibody response after the second infection (Sainsbury 2008) and gave more consistently positive qPCR results compared to skin samples scarified with tissue-culture adapted strain of SQPV despite the fact that the samples were not collected until 41 days after the second infection. It was also despite the fact that the wild-type virus inoculum apparently contained fewer virus particles than the tissue culture grown virus inoculum.

The tissues examined from the Tompkins study (§**4.2**) had been collected 6 weeks after a re-infection with SQPV when there may have been the possibility that the animals (having been infected eight months previously) were already immune to infection. Therefore a new infection study was performed. However, the results from this study were not altogether convincing as to whether or not a SQPV infection had been successfully established in the grey squirrels.

Due to the lack of antibody response 6 weeks post inoculation, as in the Tompkin's study, it was decided to re-challenge the animals and look for an anamnestic response which would suggest that the animals had indeed been primed by the first exposure to virus. However, ELISA results from all three groups (scarification, intranasal and subcutaneous infection routes) stayed below the cut off point of 0.2 OD_{450} units even after this second exposure to virus. Two of the groups, however, the iscarification (A) and intranasal (B) groups (**Figure. 4.6**) both exhibited a positive trend in terms of antibody production after the second challenge without actually rising above the 0.2 OD_{450} cut off. Interestingly, however, one squirrel (V) in group B did produce a positive ELISA reading (OD_{450} = 0.22). This animal was also qPCR positive for scent gland, eyelid skin and the scarification site. Indeed more viral DNA was detected in the eyelid skin than at the scarification site indicating the possibility of virus replication in eyelid skin. This was supported by histological findings although the changes were not specific to SQPV infection. Two squirrels in group A also showed positive qPCR results for eyelid skin, but no histopathological changes were detected. Whilst it cannot be discounted completely that some of the qPCR positive results in tissues distal to the site of infection could have been due to

mechanical transfer of the virus during grooming behaviour it is interesting that virus DNA was detected in eyelid skin, a major target tissue for SQPV in red squirrels.

The lack of a measurable antibody response in the squirrels six weeks after initial inoculation prompted fears that the inoculum had been inadequate in some way or other. Examination of it by EM (D. Everest, VLA) failed to detect any virus particles even though the qPCR had demonstrated a high content of virus DNA (10^8 genome equivalents/ml). For that reason, a new SQPV inoculum was prepared for the secondary challenge. To ensure the inoculum was relatively free of cellular contaminants (such as proteases) a single dry scab from an eyelid lesion of a naturally-infected red squirrel was used. Furthermore, in order to ensure a minimum quantity of known viable virus was present in the inoculum it was supplemented with SQPV grown *in vitro* in FLSc. Despite these measures when the tissues were collected at post mortem two weeks after the secondary challenge few were positive for viral DNA, the hoped for anamnestic antibody response was not evident in all animals and the histological examination of tissues generally did not reveal convincing evidence of viral infection.

On the positive side however, most of the animals or tissues that gave signs, histopathological or positive qPCR results, of SQPV infection came from the scarification group. Indeed scarification and topical application of virus is recognised as an appropriate route of infection for other poxviruses and so it was decided to choose this route for the time course experiment planned in **Chapter 5**.

There are key steps involved in producing a successful experimental model of infection. Firstly, the inoculum has to be infectious and contain a sufficient concentration of, in this case, virus particles to establish an infection. Secondly, the route of infection should also be appropriate for the establishment of infection. The main problem encountered in the experiments described here is that few pathological changes have ever been described in SQPV-infected grey squirrels, even those that have been naturally infected with the virus. Indeed, until recently there had been only one report of disease, associated with SQPV, in a grey squirrel (Duff *et al*., 1996). Atkin *et al*., (2010) have recently also described finding of two naturally infected animals. All these three animals had exhibited scabs to a greater or lesser degree.

Nevertheless it would appear, through the lack of reports to the contrary, that the majority of grey squirrels that are infected with the virus show few signs of being infected. Therefore infection with SQPV is most evident in red squirrels where extant signs of disease develop rapidly after exposure to the virus (Tompkins *et al*., 2002). It was not possible to obtain red squirrels to act as sentinel animals in these infection experiments to prove the virulence of the inocula and therefore it was obvious from the results obtained from this pilot study that the inocula used for further infection studies would need to be assessed in several ways to ensure that it contained viable virus.

CHAPTER 5

GREY SQUIRREL EXPERIMENTAL INFECTION: TIME COURSE EXPERIMENT

5.1 Introduction

Studying naturally occurring SQPV disease in squirrels in the wild is difficult. The mechanisms associated with virus shedding and transmission within grey squirrels and between grey and red squirrels are likely to be complex and are still poorly understood. For example, nothing is known about the duration of viral shedding, persistence and mode of viral transmission.

Results from previous chapters (**Chapter 3** and **4**) highlighted the issue that in both naturally and experimentally SQPV-infected grey squirrels, levels of viral DNA detected in different tissues analyzed was always very low, almost at the limit of Real-time PCR (qPCR) detection. This may have been because samples were not taken for analysis until several weeks post infection for the experimentally-infected animals and, as far as the naturally-infected animals were concerned, there was no way of telling how recently they had last been infected with the virus. In addition, in both experimental infections reported in **Chapter 4**, the sero-conversion in grey squirrels was not as great as had been expected from the serological data collected from naturally infected grey squirrels, where levels of serum antibody against SQPV can be relatively high (Sainsbury *et al*., 2000). The results also failed to answer the question of whether grey squirrels secrete or excrete the virus for a short time after infection, for a long time or even at all.

Although SQPV infection appears to be asymptomatic in grey squirrels (Tompkins *et al*., 2002) the subclinical signs of disease, potentially present during SQPV infection, have never been examined in detail. Previous studies mainly focused on antibody production as measured by ELISA and external clinical signs, of which there were few, if any, in grey squirrels (Tompkins *et al*., 2002; Sainsbury *et al*., 2000; Sainsbury *et al*., 2008; Sainsbury 2008; Bruemmer *et al*., 2010). Thus, in this chapter, a qualitative and quantitative time course study to monitor viral shedding,

presence of viraemia, gross pathology and histopathology in experimentally-infected grey squirrels is presented. The experiment for the first time combined a wide range of analytical techniques including a systematic clinical score and post mortem examination, ELISA, qPCR, histology and immunohistochemistry in order to progress our understanding of kinetics, location and replication of SQPV and the potential role of grey squirrels in the transmission of SQPV.

5.2 SQPV wild-type inoculum

The lack of seroconversion of the grey squirrels experimentally infected with SQPV in **Chapter 4** and the relatively low levels of virus found by qPCR had raised concerns about the viability of the virus inoculum that had been used to infect the animals. Thus before proceeding with the time course experiment described in this chapter it was decided to check the viability of the virus within the inoculum. This was made difficult by the unavailability of red squirrels that could have been used as a fully susceptible host. Instead, *in vitro* growth of the virus was used as a surrogate test for proving viability.

5.2.1 Quantitation of viral DNA within the inoculum

The amount of viral DNA was calculated from total DNA extracted from 30 µl of the SQPV wild-type inoculum using a qPCR standard curve (**Figure 5.1**). The limit of the detection of the assay was determined from serial dilutions of SQPV cosmid #86 (§**2.5.7.1**). The qPCR results from the serial dilution of SQPV cosmid #86 DNA showed a linear correlation with a dynamic range of eight orders of magnitude ranging from approximately 3 to 3 x 10^7 genome copies (**Table 5.1**).

The assay reproducibly detected up to 0.13 fg of target sequence which corresponds to 3 genome equivalents/reaction.

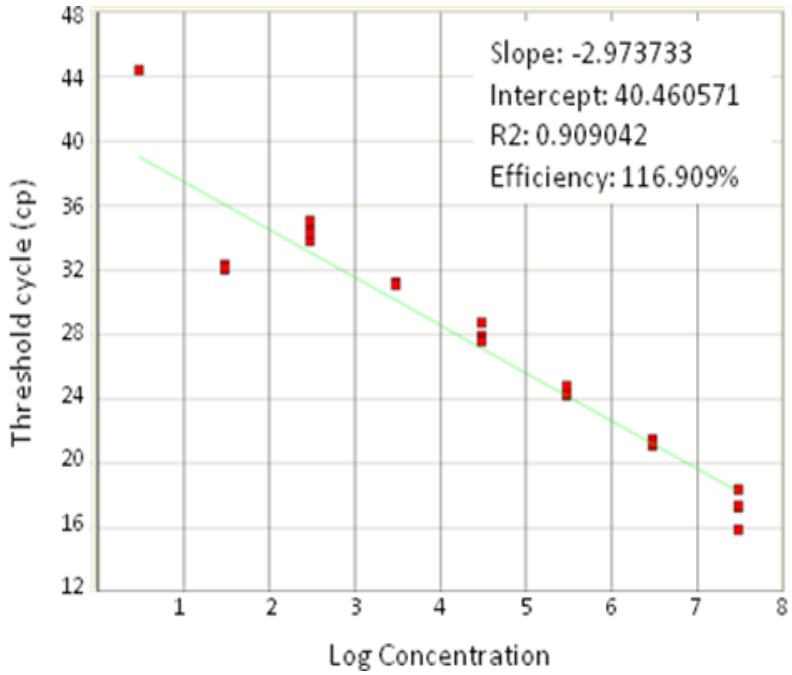


Fig. 5.1 Standard curve using serial dilutions of SQPV cosmid #86 to quantify viral DNA in the SQPV inoculum.

The linear regression analysis is calculated from C_p values against the Log concentration. SQPV cosmid #86 concentration used to perform the standard curve ranged between 1.3 ng/ul for the starting dilution (3 x 10^7 copies of target gene) to 0.13 fg/ul (3 copies of the target gene).

Four replicates of the SQPV wild-type inoculum had Cp values that ranged from 20.5 to 21.0 corresponding to a mean of 3 x 10^7genome equivalents. Therefore the final concentration in the SQPV inoculum was calculated as 5 x 10^{10} genes copies/ml.

Table 5.1 Mean C_p value and C_p range obtained from serial dilutions of SQPV cosmid#86 used to quantify the SQPV DNA in the wild-type inoculums.

Detector	Genomes equivalent/reaction	C_p mean	C_p range
SQPV I1L	3x 10^7	19.4	18.2-20.3
SQPV I1L	3x 10^6	23.2	22.9-23.3
SQPV I1L	3x 10^5	26.3	26.1-26.7
SQPV I1L	3x10^4	29.8	29.3-30.6
SQPV I1L	3x10^3	32.9	32.8-32.9
SQPV I1L	300/30	35.2	33.8-37

Mean C_p value and C_p range corresponding to qPCR standard curve using I1L detector to amplify a fragment of SQPV DNA derived from Cosmid #86. Four replicates were conducted for each dilution.

5.2.2 SQPV growth in culture of FLSc

The infectivity of the inoculum was tested by inoculating foetal lamb skin cells (FLSc) with the wild-type virus preparation (§**2.3.1.2**). Even if no visble signs of infection were present initially, the supernatant from the inolculated cells were passaged on to fresh monolayers of FLSc. SQPV produced visible cytopathic effects (CPE) in the infected cells at the seventh passage *in vitro*, assessed three days post infection. Infected cells showed typical CPE with cell rounding and retraction from the substrate leaving several gaps in the monolayer, while in contrast the mock infected cells did not show any CPE (**Figure 5.2**).

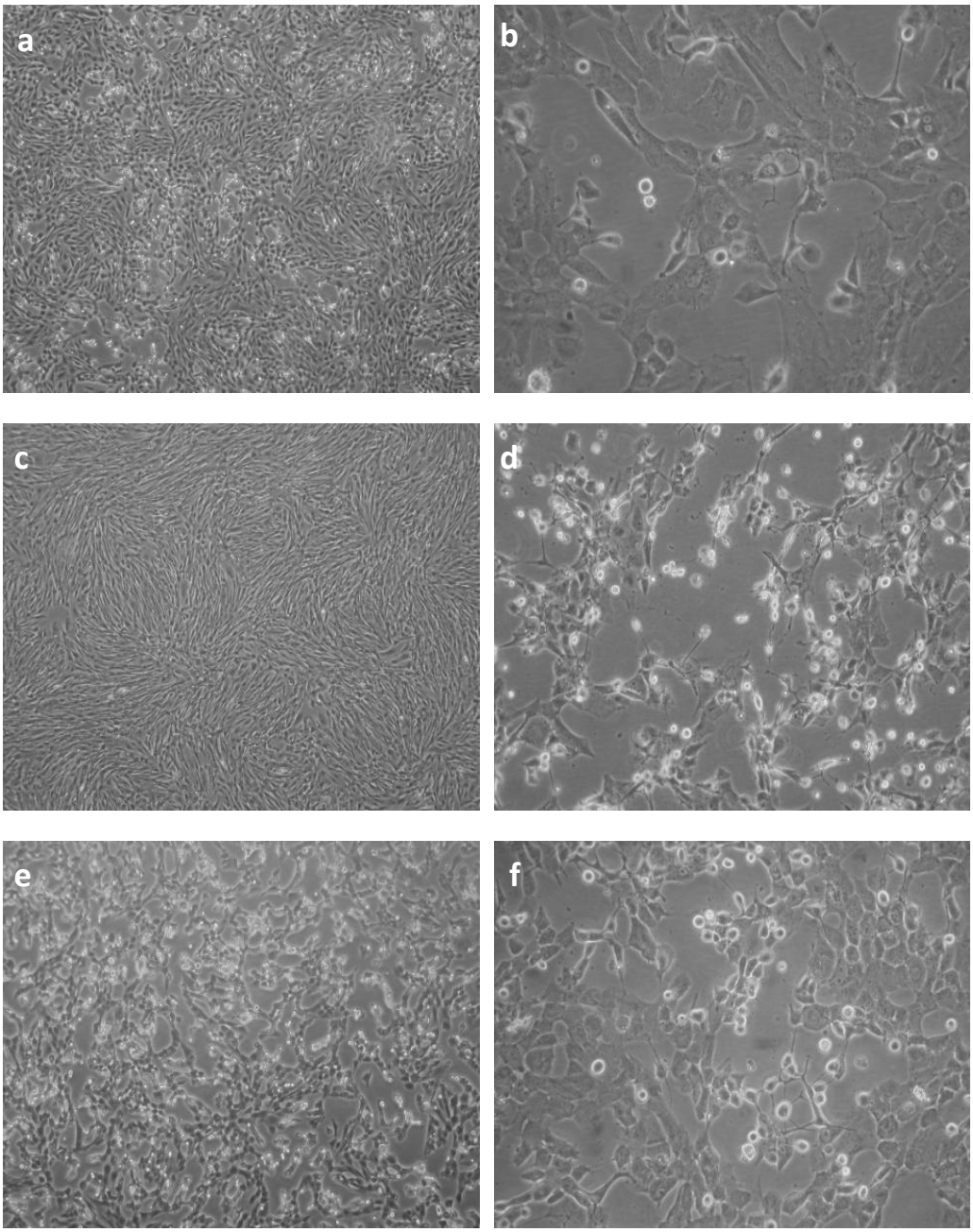


Figure 5.2. The cytopathic effect (CPE) found in cultures of foetal lamb skin (FLSc) infected with the crude type SQPV inoculum showing cell rounding.

Confluent FLSc monolayers were infected with a crude-type SQPV inoculum. **(a)** Infected culture at 3 days post infection (DPI) after the seventh passage of SQPV in culture. **(b)** Image of CPE at higher magnification than in image (a). **(c)** Mock infected cells at 3 DPI. **(d)** Infected culture at the final stage (6 DPI), seventh passage of SQPV in culture, showing marked CPE. **(e)** Infected cells at 1 DPI after the eighth passage of SQPV in culture showing moderate CPE. **(f)** CPE at higher magnification than in image (e). Cells were observed using an inverted phase-contrast microscope.

5.2.3 SQPV detection using EM

Having determined the presence of viral DNA within the inoculum by qPCR and the infectivity of the virus by growth and passage *in vitro*, the morphological integrity of the SQPV particles were evaluated by electron microscopy (courtesy of David Everest, VLA, Weybridge). The presence of intact SQPV particles was confirmed (**Figure 5.3**).

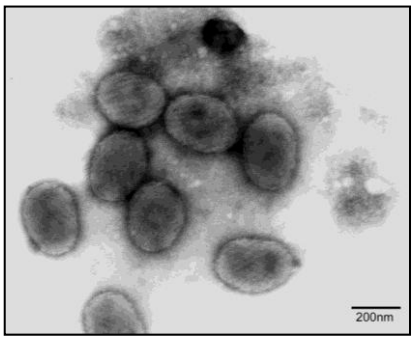


Fig. 5.3 SQPV particles identified by E.M. from the virus inoculums.

Image courtesy of D.Everest, VLA (Weybridge, Surrey, UK)

In order to confirm that the virus-associated CPE in infected cells was due to SQPV and not any other virus that may have been present in the inocula, cell debris and supernatant from infected cells taken at day six post infection was examined by EM and the presence of SQPV confirmed (**Figure 5.4**)

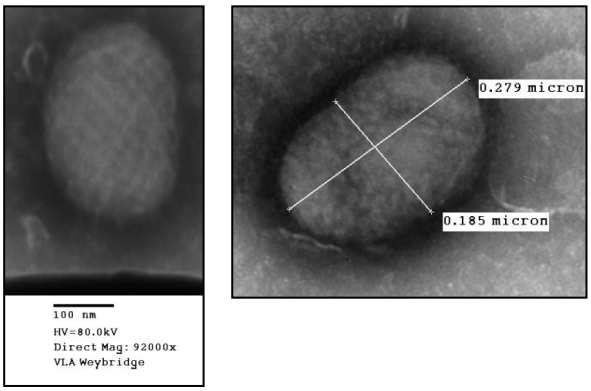


Figure 5.4 SQPV particles identified by EM from virus inoculum grown in foetal lamb skin cells.

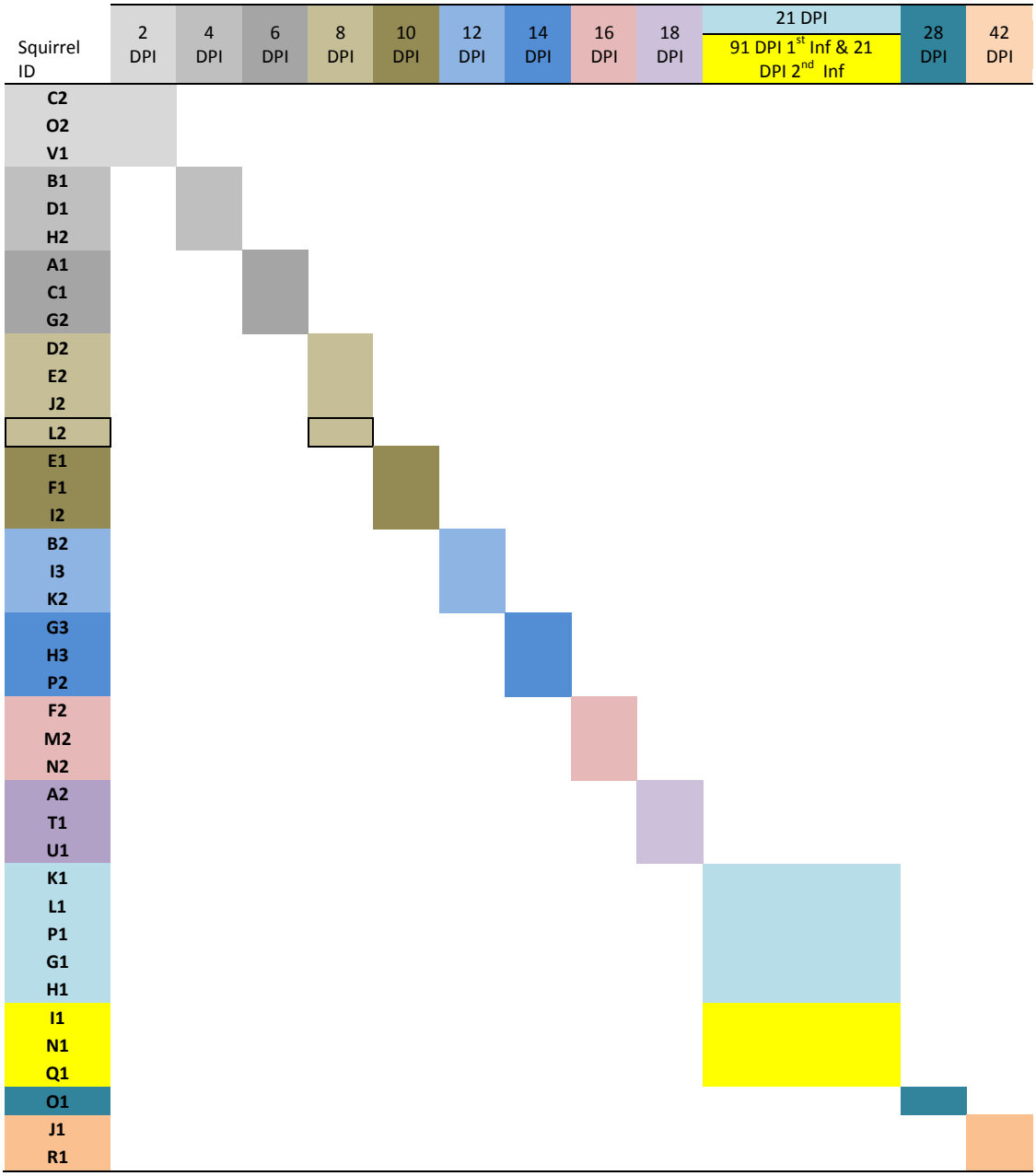
Image courtesy of D. Everest, VLA (Weybridge, Surrey, UK).

5.3 Study design

Thirty-eight grey squirrels were experimentally challenged via skin abrasion with 0.2 ml of SQPV wild type inoculum. The experimental methods used are detailed in **Chapter 2**. Prior to the start of the experiment, blood samples were collected from each individual in order to confirm their serological status with respect to anti-SQPV antibodies. The grey squirrels were divided randomly by gender and estimated age into 13 experimental groups (12 groups of three and one group of two individuals). An additional 39th animal (squirrel L2, female) arrived with an injured leg and was therefore excluded from the experimental infection and used as a control.

The experimental groups were defined by the sequence in which the animals were sacrificed following the SQPV challenge. The group of two animals together with another group of three was euthanized 21 days post infection. A single animal from another group died naturally under anesthesia 28 days post infection and is therefore listed separately. The remaining two animals in this group were sacrificed 42 days post infection. The single squirrel (L2) used as a control was included in the group of three animals sacrificed 8 days post SQPV challenge and was mock scarified. All the other animals were scarified at least once on the hind limb with the SQPV inoculum (see §**2.6.5**). One group of three animals was challenged a second time, after 10 weeks, and sacrificed after 91 days (21 days after the second infection). A schematic representation of the time course experiment is provided in **Table 5.2**.

Table 5.2 Schematic table of the time course experimental infection for 39 grey squirrels.



The top coloured row indicates the days post infection (DPI). Squirrel L2 was not infected. Each coloured square represents the time point when animals were euthanized. Animals that were re-infected (Inf) are indicated by the colour yellow.

The experimental infection covered two time frames: the acute phase of infection, with animals being euthanized from two to 18 days post-infection (DPI) and a longer term infection from 21 to 42 days post infection. Hereafter, for ease of presentation of the results, the terms “short term infection” and “long term infection” will be used to refer to the two time periods covered by the experiment.

5.4 Clinical assessment of squirrels including body weight and clinical score

5.4.1 Short term infection

The previous experiment (§**4.3.2.1**) clearly showed that the body weight of SQPV-challenged grey squirrels did not change markedly as a result of infection. For the purpose of the short term experiment squirrels were therefore only weighed on day zero (challenge day) and on the end day for each group just before animals were euthanized.

Figure 5.5 shows the mean percent of body weight change for each group pre-infection and on the day the animals were sacrificed. There were no considerable body weight losses in any of the nine groups with none of the animals showing a weigh lost ≥ 30% according to the clinical score. In fact eight out of nine groups showed an increase in body weight during the course of the experiment.

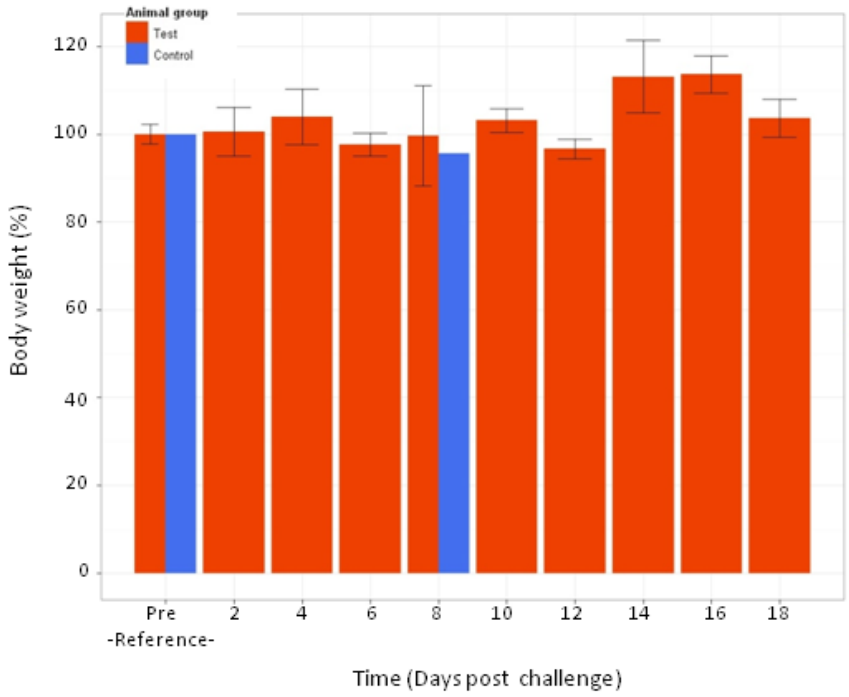


Figure 5.5 Body weight (%) fluctuation during the course of the short term infection.

Body weight (%) for each group of squirrels recorded at two day intervals as compared to the pre-challenge value shown in the first red and blue column (Pre-Reference-). Error bars represent 95% Confidence Intervals. In blue is the uninfected female squirrel L2 (control) and in red infected animals (test).

The same clinical scoring system (§**2.6.4**), as used in **Chapter 4** and based on the one utilized to assess the impact of squirrelpox disease on red squirrels (Tompkins *et al*., 2002), was used to assess the grey squirrels following the SQPV challenge. The daily clinical score for each individual ranged between zero and a maximum of three on a single occasion. Recorded positive scores were predominantly associated with food consumption behaviour (loss of appetite). **Table 5.3** shows an overview for all groups for the short term infection experiment. A count of all daily scores across all individuals and groups gives a median score of zero. The results demonstrate that animals did not display any marked visible clinical signs or physical distress. There was a temporal progression in clinical scores post infection, with most of the positive scores (>80%) in the period from day three until day 12 post infection (**Table 5.3**).

Table 5.3 Clinical scores, short term experiment.

DPI	C2	O2	V1	B1	D1	H2	A1	C1	G2	D2	E2	J2	L2	E1	F1	I2	B2	I3	K2	G3	H3	P2	F2	M2	N2	T1	U1	A2
1			3																2									
2									1				1															
3										1		1	1				1							1				
4																												
5								1	1																			
6													1	1			1		1									
7																	1	1										
8																												
9																	1	1	2			1						
10																	1		2			1						
11																	1											
12																												
13																												
14																												
15																								1				
16																												
17																												
18																												

Daily clinical scores post infection for each group based on defined assessment criteria (the Home Office Licence stipulated that animals reaching a score of eight or more on three consecutive occasions would be euthanized). The first row indicates the squirrel ID and first column the days post infection (DPI). For the purpose of illustration, only positive values are shown – blank spaces indicate a clinical score of zero. The end point for each group is shaded in grey.

Figure 5.6 shows the group (squirrels B2, I3 and K2) which received the highest number of positive scores (range zero to two) in that period.

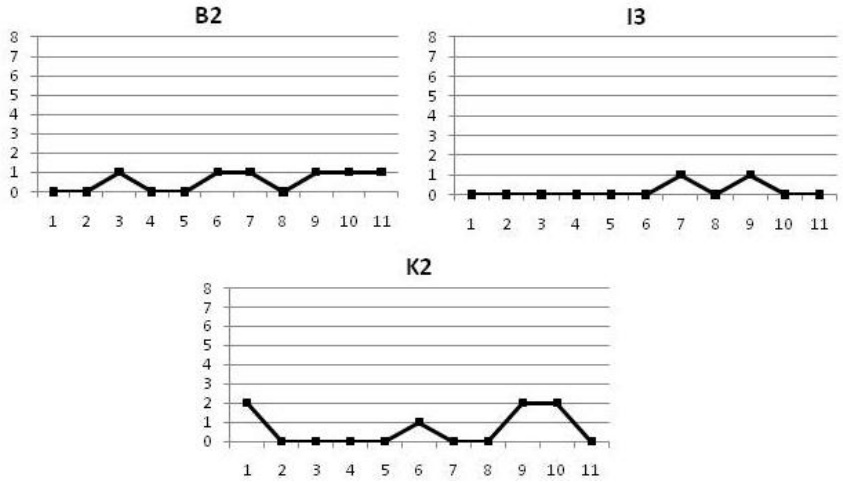


Figure 5.6 Example plots of three individual clinical scores.

Illustration of the group (B2, I3, K2) that received 46% of all positive scores. This group was sacrificed 12 days post-challenge. The *x*-axis shows days post infection, the *y*-axis clinical score (scale 0-8). A score of eight indicates the defined humane end point at which infected grey squirrels would be removed from the experiment.

5.4.2 Long term infection

The identical clinical scoring system as used for the short term infection was used to assess the grey squirrels for the long term infection study. However, it was not technically possible to weigh the squirrels on their own without anaesthesia. Therefore, instead, the squirrels were weighed in their nest boxes every three days. In addition they were weighed individually on the day of blood sampling and when challenged or euthanized (as the red numbers indicated in **Table 5.4**). At no time did any squirrel lose more than 10% of their starting weight when first captured from the wild. In **Figure 5.7** the average body weight for the group of challenged animals is represented in red bars for each week post infection and the blue bars represent the mean of the two control animals (H1 and G1) which were challenged for the first time 10 weeks after the start of the experiment (day zero). At the same time the remaining three squirrels (I1, N1 and Q1) were re-challenged.

Table 5.4 Clinical score system long term experiment.

	K1	L1	P1	O1	J1	R1	G1	H1	I1	N1	Q1
1											
2			3								
3	1				1						
4									2		
5			3		1		2				
6											
7											
8	3		3		2						
9			1								
10											
11											
12			3								
13			3								
14			1			1		1			
15					1			1			
16											
17		2	2								
18											
19	4	6	6								
20											
21		2	1				1				
22					1						
23											
24							1				
25											
26				1							
27											
28											
29											
30											
31							1				
32											
33											
34								1			
35						1					
36											
37											
38											
39					1	1					
40											
41						1					1
42											
43					1		1				
44											
45											
46								1			
47										1	
48											
49											
50											
51											
52											
53											
54											1
55											
56							1				
57							1				
58							1				
59							1		1		1
60											
61											
62											
63											
64								1			
65											
66								2		1	
67							1				1
68									1	1	
69							2				
70							1		1	1	
71											1
72							1				
73							1	1			1
74								2			
75											
76											1
77							2				
78							2	1			1
79							1		1		
80									1		1
81									1		
82							1				1
83							1				1
84											
85											
86							1				1
87											1
88											
89											1
90											1
91											

Daily clinical scores post infection (DPI) for each group based on defined assessment criteria. The dotted black line after 70 days indicates the first challenge for squirrels G1 and H1 and the SQPV re-challenge for animals I1, N1 and Q1. Red numbers represent the days on which blood samples were collected. The first row indicates the squirrel ID and the first column DPI. Only positive values are shown – blank spaces indicate a clinical score of zero. The end point for each group is shaded in grey.

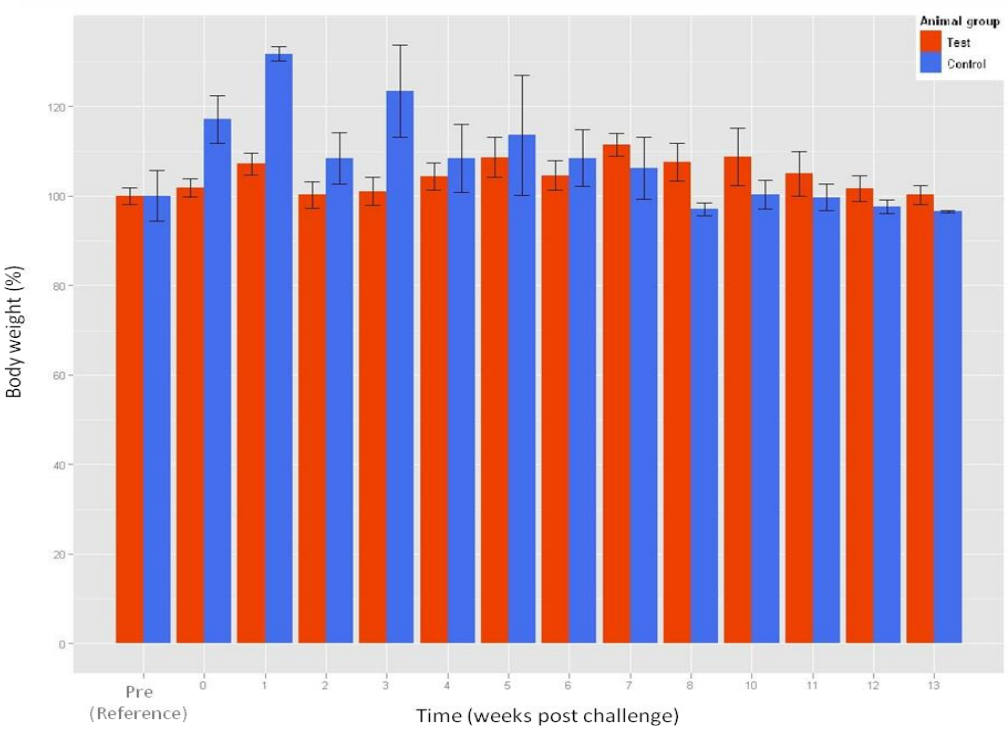


Fig 5.7 body weight (%) fluctuation during the course of the long term infection.

Change in the body weight (%) recorded weekly as compared to the pre-challenge value shown in the first red and blue column (Pre-Reference-). Error bars represent 95% Confidence Intervals. Red bars (test) are animals challenged twice, first at time zero (0) and again at week 10. Animals H1 and G1 that were only challenged once (at week 10) are indicated in blue (control).

Neither the first nor the second SQPV challenge (of three animals: I1, N1 and Q1) led to any observable pattern in clinical scores. The clinical score assessment (**Table 5.4**) for the entire length of the experiment (91 days) showed no clear or consistent trend. Scores ranged from zero to six, but the latter occurred only on two occasions for two different animals, although both were in the same group. The high values were due to a combination of loss of appetite and actual weight loss in the two individuals. However, this was only temporary and scores improved for both animals. The animal (P1) which scored consistently in the clinical assessment arrived with a prior minor injury which led to a scar on its nose possibly explaining its persistent scoring.

5.5 Serology

5.5.1 Short term infection

For the short term infection blood samples were collected before the start of the experiment in order to confirm the absence of antibody against SQPV in the grey squirrels. A second blood sample was collected on the final day for each individual group. In **Table 5.5** ELISA OD_{450} values are shown for the pre-infection bleed and for the appropriate days post infection for each group. Only two squirrels (B2 and N2, highlighted in bold in **Table 5.5**) out of 27 (the 28^{th} animal L2 was not infected) seroconverted during the 18 days post infection with SQPV. Both had an ELISA OD_{450} reading of slightly above 0.20 which is regarded as the threshold that determines positivity. It is worth noting that both animals that tested positive did so after 12 and 16 days suggesting a time-lag for an antibody response to occur. However, since no continuous sampling was performed, the precise day for antibody conversion is therefore not known. It was interesting that squirrel B2 had given a reading of OD_{450} 0.121 for its pre-infection bleed. This was higher than the readings from all the other animals (it was the only squirrel that gave a pre-infection reading >0.1), but is still officially regarded as a negative reading.

Table 5.5 Individual anti-SQPV antibody ELISA OD_{450} values for the short term infection.

Squirrel ID	Mean ELISA OD pre-infection	Squirrel ID	Days post infection	Mean ELISA OD post infection
V1	-0.013	V1	2	0.009
C2	0.033	C2	2	-0.064
O2	0.005	O2	2	0.001
B1	-0.013	B1	4	0.013
D1	-0.018	D1	4	0.006
H2	-0.002	H2	4	0.005
A1	-0.047	A1	6	0.008
C1	-0.011	C1	6	0.007
G2	0.027	G2	6	0.031
D2	0.002	D2	8	-0.010
E2	0.012	E2	8	0.024
J2	-0.078	J2	8	-0.017
L2	0.047	L2	8	0.040
E1	-0.003	E1	10	0.004
F1	-0.018	F1	10	0.015
I2	0.009	I2	10	0.004
B2	**0.121**	**B2**	**12**	**0.224**
I3	0.020	I3	12	0.029
K2	0.007	K2	12	-0.002
G3	0.021	G3	14	0.069
H3	-0.002	H3	14	-0.009
P2	0.013	P2	14	0.037
F2	0.007	F2	16	-0.005
M2	0.018	M2	16	0.038
N2	**-0.001**	**N2**	**16**	**0.219**
T1	0.017	T1	18	0.026
U1	-0.011	U1	18	-0.006
A2	0.088	A2	18	0.038

Anti-SQPV antibody ELISA values for 28 grey squirrels for the short term infection. Squirrel L2 was housed in the same room with the other infected animals but was not challenged. The two squirrels that seroconverted are shown in bold.

5.5.2 Long term infection

For the long term infection blood samples were again collected before the start of the experiment in order to confirm the absence of antibody against SQPV in the grey squirrels and subsequently every two weeks and at the end point for each group (**Tables 5.6** and **5.7**). For the long term experiment two animals (G1 and H1) were kept as controls and they were not challenged until after 10 weeks post infection at the same time as the second challenge for one of the other groups.

Table 5.6 Mean anti-SQPV antibody ELISA OD results for long term infection.

squirrels per group	**9**	**9**	**3**	**6**	**5**	**3**	**3**	**3**	**3**	**3**
Time PI	pre	2w	3w	4w	6w	8w	10 w second challenge	1w	2w	3w
ELISA OD	0.000	0.043	0.037	0.027	0.061	0.019	0.029	0.235	0.278	0.302

squirrels per group (control)	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**
Time PI	pre	pre	pre	pre	pre	pre	first challenge	1w	2w	3w
ELISA OD	-0.010	-0.014	-0.014	0.000	0.010	0.010	-0.009	0.012	0.010	0.173

Summary of anti-SQPV antibody ELISA results showing mean OD_{450} values for each group. Numbers of animals per group and blood sampling time pre and post infection are given. Time is shown in weeks (w) post infection (PI).

Table 5.7 Individual anti-SQPV antibody ELISA OD_{450} values for long term infection.

squirrel	comment	weeks PI	DPI	ELISA OD
G1		PRE		-0.008
H1		PRE		-0.012
I1		PRE		0.005
J1		PRE		-0.004
K1		PRE		0.025
L1		PRE		-0.005
N1		PRE		0.016
O1		PRE		0.005
P1		PRE		-0.026
Q1		PRE		0.014
R1		PRE		-0.028
G1		PRE		0.011
H1		PRE		-0.039
I1		2	14	-0.005
J1		2	14	0.055
K1		2	14	0.016
L1		2	14	0.032
N1		2	14	0.098
O1		2	14	0.036
P1		2	14	0.001
Q1		2	14	0.095
R1		2	14	0.062
K1		3	21	0.073
L1		3	21	0.053
P1		3	21	-0.015
G1		PRE		-0.001
H1		PRE		-0.008
I1		4	28	0.068
J1		4	28	0.041
N1		4	28	0.094
O1		4	28	-0.001
Q1		4	28	0.034
R1		4	28	0.008
G1		PRE		-0.004
H1		PRE		0.004
I1		6	42	0.044
J1		6	42	0.073
N1		6	42	0.058
Q1		6	42	-0.038
R1		6	42	0.014
G1		PRE		-0.005
H1		PRE		0.024
I1		8	56	0.011
N1		8	56	0.026
Q1		8	56	0.020
G1	1st challenge	PRE		0.005
H1	1st challenge	PRE		-0.056
I1	2nd challenge	10	70	0.055
N1	2nd challenge	10	70	0.011
Q1	2nd challenge	10	70	0.022
G1		1	7	0.017
H1		1	7	0.007
I1		11+1	77+ 7	0.165
N1		**11+1**	**77+ 7**	**0.474**
Q1		**11+1**	**77+ 7**	**0.200**
G1		2	14	0.060
H1		2	14	0.109
I1		12+ 2	84+14	0.179
N1		12+ 2	84+14	**0.364**
Q1		12+ 2	84+14	**0.291**
G1		**3**	**21**	**0.258**
H1		3	21	0.088
I1		**13+ 3**	**91+21**	**0.247**
N1		**13+ 3**	**91+21**	**0.326**
Q1		**13+ 3**	**91+21**	**0.333**

Repeated ELISA OD_{450} values for 11 grey squirrels throughout the course of the long term infection. Values regarded as seropositive are shown in bold.

All animals were confirmed negative at the start of the experiment (all ELISA OD_{450} readings <0.03). The second blood sample was collected two weeks post-challenge from all 11 squirrels (nine infected and two controls). None showed an antibody response to SQPV infection. Repeated blood sampling and serological analysis did not indicate any sero-conversion up to the 10^{th} week post infection. As a result, three animals were re-challenged (I1, N1 and Q1) following the same infection protocol as before. The control group (G1 and H1) was also challenged at this point with SQPV for the first time. **Figure 5.8** shows the mean ELISA OD_{450} value for both groups plotted against time post infection.

For graphical representation and homogeneity, the ELISA resulted values were plotted as a mean ELISA for each group. The test group, red line, consisted of a number of animals ranged between N= 9 and descending to N=3 during the course of the experiment while the control group, blue line, was represented by only two animals during the whole length of the experiment. Even if the ELISA mean result of the control group did not excited the cut off value of 0.2, it is worth to note, as also shown in **Table 5.7**, that one of the two squirrels (G1) did seroconvert giving a ELISA OD_{450} reading = 0.258.

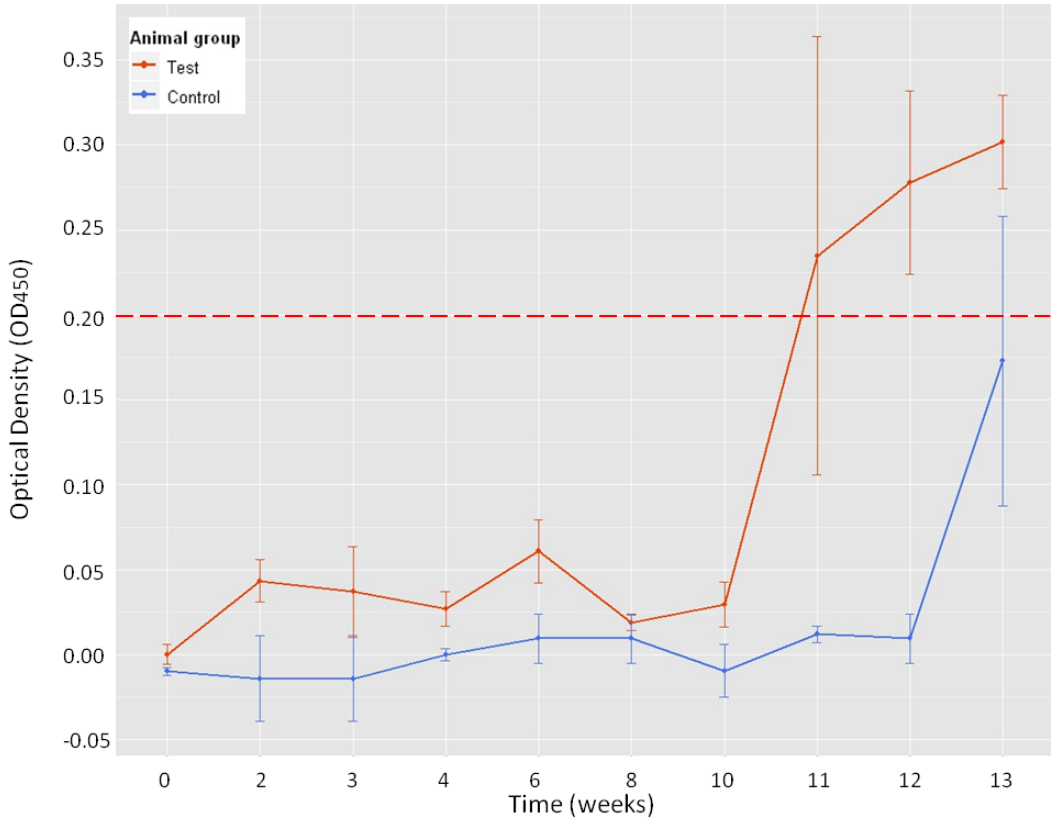


Fig. 5.8 Anti-SQPV antibody ELISA OD_{450} values for long term infection.

Corrected optical density (OD_{450}) mean values with 95% Confidence Intervals for a direct ELISA testing for the presence of antibody against SQPV in sera collected from animals (red line) challenged by scarification at day zero (pre infection time) and animals used as control group (blue line) that were sham-infected with mock inoculum. Animals from the group labelled 'test' were infected twice with the second challenge occurring 10 weeks after the first. Animals from the test group were also challenged with SQPV for the first time at week 10. The dashed red line represents the ELISA cut off value of 0.20.

Two out of three squirrels in the group that was challenged for the second time showed an immediate antibody response to SQPV and all three tested ELISA positive by week three after the second infection (**Table 5.7** and **Figure 5.8**). It was interesting that squirrel G1 of the previous control group that was challenged for the first time had seroconverted by week three while the other (H1) remained negative. Most ELISA data published in the literature are snapshots obtained and it is worth noting that ELISA OD_{450} values of individuals fluctuated slightly over the three week period.

5.6 Post Mortem examination

For both the short and long term infection, post mortem examination was carried out on all squirrels from each of the different groups at their respective end points. The animals were weighed prior to euthanasia. They were examined for external lesions and body measurements were taken such as crown-rump length (CRL) and shin length (SL) (**Table 5.8**). In addition, an examination for internal pathological changes was performed and samples collected for further analysis (qPCR, histopathology and immunohistochemistry). The liver and spleen were weighed and the spleen was also measured in order to detect potential signs of viral infection. Details of protocols followed are given in §**2.7**.

5.6.1 Short term infection

Between two and 18 DPI, skin lesions present at the site of SQPV scarification showed progressive healing. **Figures 5.9-A-** and –**B-** show representative examples of skin lesions observed at different times post infection from this period. Between two and eight DPI in all animals, the skin at the site of SQPV scarification was characterized by a mild exudative erythematous dermatitis with a haemorrhagic sero-cellular crust. In contrast, in the mock scarified contra-lateral limb, the sero-crust was generally no longer evident after four DPI. By 10 DPI a mild scab was present in some individuals with a mild thickening of the skin in the area of SQPV challenge and the haemorrhagic sero-cellular crusts had disappeared (e.g. **Figure 5.9-B- g** and **h**). Between 14-18 days post scarification lesions become less visible and differences between mock scarified (on the right side of the picture, left medial thigh, **Figure 5.9-B-**) and SQPV challenged limb (left side of the pictures, **Figure 5.9-B-**) were less demarcated. By 18 DPI, the infected lesions were considerably less apparent and had healed progressively (**Figure 5.9-B- j**). The scarified skin from a mock infected squirrel (L2) at eight DPI is also presented in order to compare the skin lesion due to mechanical injury caused from the scarification procedure with the actual skin lesions of the infected squirrels (**Figure 5.9-A-d**).

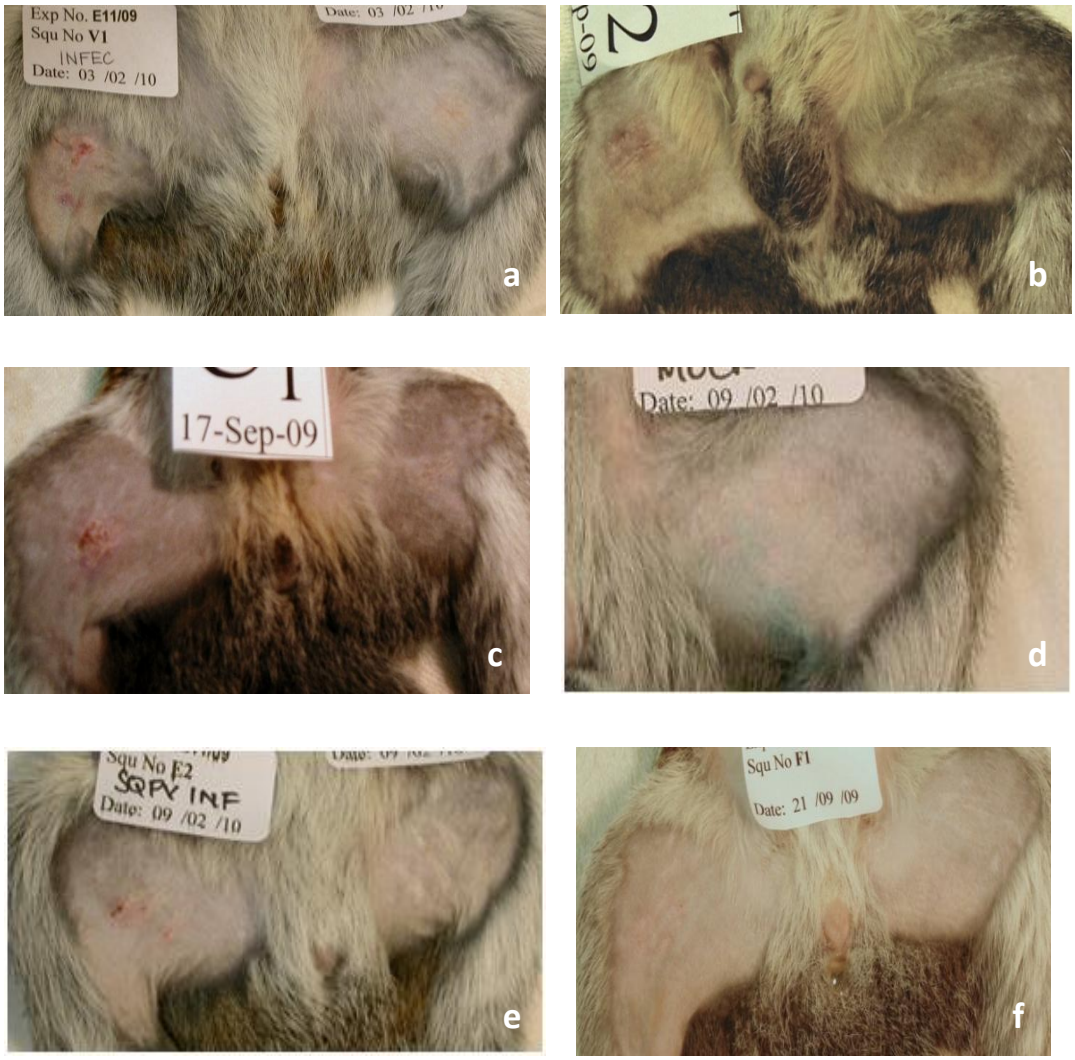


Figure 5.9-A- Progression of clinical healing of scarified thigh skin from two to 10 DPI.

Site of SQPV challenged scarified skin was the right medial thigh (on left side of figures) and left medial thigh was mock challenged. **(a)** Squirrel V1, 2 DPI; **(b)** Squirrel H2, 4 DPI; **(c)** Squirrel C1, 6DPI; **(d)** Squirrel L2 mock, 8 DPI; **(e)** Squirrel E2 8 DPI; **(f)** Squirrel F1, 10 DPI.

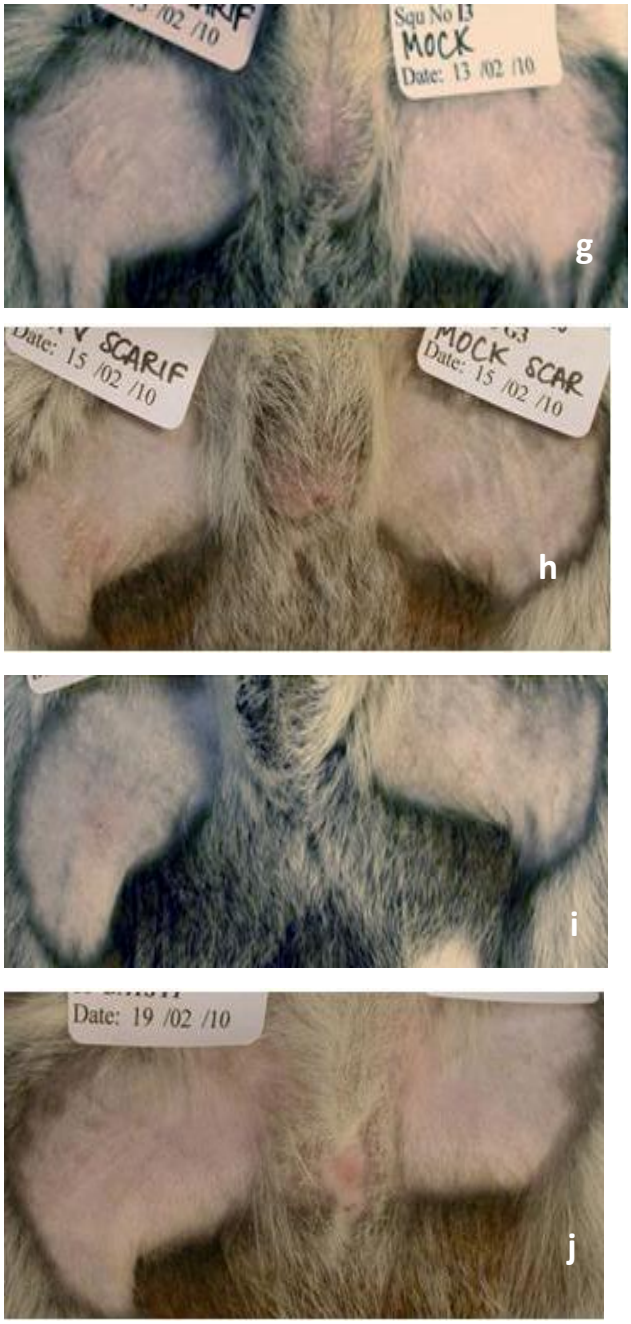


Figure 5.9-B- Progression of clinical healing of scarified thigh skin from 12 DPI to 18 DPI.

Site of SQPV challenged scarified skin was the right medial thigh (on left side of figures) and left medial thigh was mock challenged. **(g)** Squirrel I3, 12 DPI; **(h)** Squirrel G3, 14 DPI; **(i)** Squirrel N2, 14 DPI; **(j)**: A2, 18DPI

In **Table 5.8** an index [body weight (g)/ (CRL/SL)] was calculated in order to correct observed body weights in relation to body size and to objectively compare individual animals. There was no consistent pattern of weight loss or gain in relation to corrected size. In total, 18 out of 28 animals experienced an increase in body weight and 10 animals a weight loss. However, weight gains were much larger than weight losses (%) with the maximum weight gain of 30.28% and the greatest weight loss of only 8.07%.

Table 5.8 Short term infection biometric data and difference in body weight (%) at the end of experiment.

Squirrel	Gender	Crown-rump (CRL) (mm)	Shin length (SL) (mm)	Crown-rump/shin length	Body weight(g) end point experiment	Index	Body weight fluctuation (%)
C2	F	197	80	2.5	499	199.6	-0.8
O2	M	210	85	2.5	604	241.6	-8.07
V1	F	200	85	2.4	573.4	238.9	+0.77
B1	F	210	80	2.6	569	218.8	+2.56
D1	M	215	85	2.5	643	257.2	+2
H2	M	215	75	2.9	521.4	179.8	-0.13
A1	M	210	85	2.5	571.4	228.6	+7.24
C1	M	195	75	2.6	527	202.7	+0.82
G2	F	210	80	2.6	529.3	203.6	-4.6
D2	M	210	75	2.8	639.5	228.4	-1.36
E2	F	180	80	2.3	429.6	186.8	+1.66
J2	M	195	80	2.4	591.9	246.6	+2.58
L2	F	210	80	2.6	591.4	227.5	-4.34
E1	M	215	80	2.7	602.8	223.3	+14.75
F1	F	210	75	2.8	558.4	199.4	+0.99
I2	M	220	75	2.9	558	192.4	+3.89
B2	F	195	78	2.5	554.2	221.7	-6.92
I3	M	180	80	2.3	544.6	236.8	+5.01
K2	F	195	82	2.4	512.6	213.6	+4.19
G3	M	190	85	2.2	704.4	320.2	+11.46
H3	M	190	75	2.5	545.4	218.2	-4.32
P2	F	200	77	2.6	636	244.6	-0.39
F2	M	185	75	2.5	590.6	236.2	+19.12
M2	F	210	82	2.6	632	243.1	-1.1
N2	M	200	80	2.5	672.1	268.8	+5.84
T1	F	190	73	2.6	551	211.9	+9.54
U1	M	200	80	2.5	623.7	249.5	+13.4
A2	F	190	73	2.6	553.7	213.0	+30.28

Squirrels biometric data collected during the post mortem analisys. Calculated body index which allows a comparison of different individuals and has been corrected for body size (body weight (g)/ (CRL/SL)); individual body weight (g) at the end of the experiment and body weight fluctuations expressed as a percentage change (start weight compared to the weight at end of experiment) over the course of the short term experiment.

Table 5.9 Liver and spleen measurements in the short term infection.

Squirrel ID	Gender	Liver weight (g)	Spleen weight(g)	Spleen length (mm)	Spleen width (mm)
C2	F	17	2.3	50	17
O2	M	18.4	1.1	47	11
V1	F	17.1	1.8	53	16
B1	F	22.7	1.4	47	15
D1	M	18.5	1.1	42	13
H2	M	14.2	4.5	45	14
A1	M	18.3	1.1	32	14
C1	M	16.9	0.7	36	11
G2	F	13.5	2.5	55	18
D2	M	14.1	1.7	56	17
E2	F	9.6	1.3	41	14
J2	M	13.2	1.6	47	15
L2	F	14.1	1.3	44	13
E1	M	21.1	2.2	46	15
F1	F	13.6	0.9	46	11
I2	M	18.9	1.8	51	16
B2	**F**	**12.1**	**2.2**	**60**	**15**
I3	M	14.4	1.9	50	17
K2	F	12	1.7	45	16
G3	M	20.6	1.5	50	12
H3	M	13.2	1.8	50	13
P2	F	16.9	0.8	/	/
F2	M	19.3	1.6	50	15
M2	F	14.9	1	38	11
N2	**M**	**15.2**	**1.8**	**48**	**15**
T1	F	13.8	1.8	52	16
U1	M	15.4	1.8	47	16
A2	F	17.4	3	55	20
MEAN		15.94	1.72	47.52	14.67

Liver and spleen weights and spleen size (length and width) for 28 squirrels in the short term infection experiment. SQPV ELISA positive squirrels are indicated in bold.

Spleen weight varied from 0.7-4.5 g with a mean of 1.72 g. There was no significant correlation between ELISA positive status of individuals and spleen weights, (see **Table 5.5** and **5.9**; Pearson's correlation: r=0.0398, N=28, p=0.841) although ELISA positive B2 had the longest spleen. However, the weight did not differ significantly from the mean and there were no macroscopic pathological changes.

Liver weight of the 28 squirrels ranged from 9.6-21.1 g with a mean of 15.94 g. The smallest animal was E2 (**Table 5.8** index value) and this may explain the small liver weight. **Figure 5.10** shows histograms and Kernel density distributions for each of the values provided in **Table 5.9**.

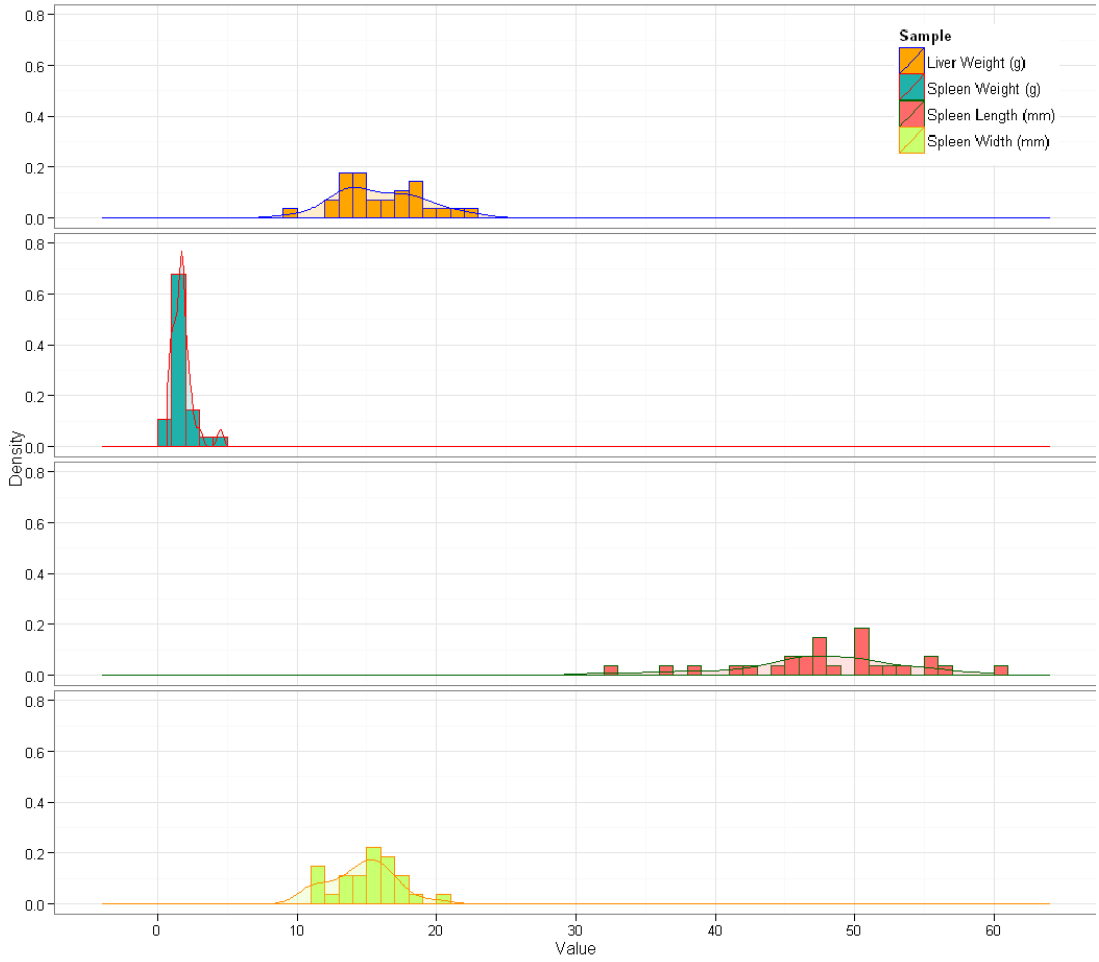


Figure 5.10 Histograms with Kernel density estimation of liver /spleen weights (g), spleens' length and width (mm) of 28 grey squirrels measured during the post mortem examination.

Histogram and Kernel density distribution are represented for the liver's weight (orange bars and blue line) in the first row and for the spleen (dark green bars and red line) in the second row; spleen length (red bars and green line) in the third row and spleen width (light green bars and orange line) in the last row. Density is plotted against value in grams (g) for the weight and millimetres (mm) for the length and width of the spleen.

An unidentified hepatic parasite (**Figure 5.11**) was found in the liver of squirrel E1 and the increased weight (21.1 g) compared to the mean liver weight of the group (15.94 g) could be linked to the presence of the parasitic infection. In addition this animal showed an enlargement of the brachial lymph node compared to the other squirrels examined.

Figure 5.11 Post mortem of squirrel E1.

Liver with parasite is indicated by blue circle and enlarged brachial lymph node by yellow circle.

5.6.2 Long term infection

Similar to the results of the short term infection, in the longer term study there were few clinical signs of infection of the skin in the SQPV challenged area other than a mild dermatitis during the first week. In **Figure 5.12** the progression of the lesions at two, four and six weeks after infection is shown for four different squirrels. At two weeks post infection a very mild dermatitis was still noticeable. After four weeks fur had started to re-grow at the site of the skin lesion and by six weeks the lesion was completely covered by new fur in most of the animals. Of interest was the pattern of re-growth in that fur grew back faster at the site of the lesion compared to the shaved surrounding area (squirrel R1 **Figure 5.12**). The reasons for this are unclear.

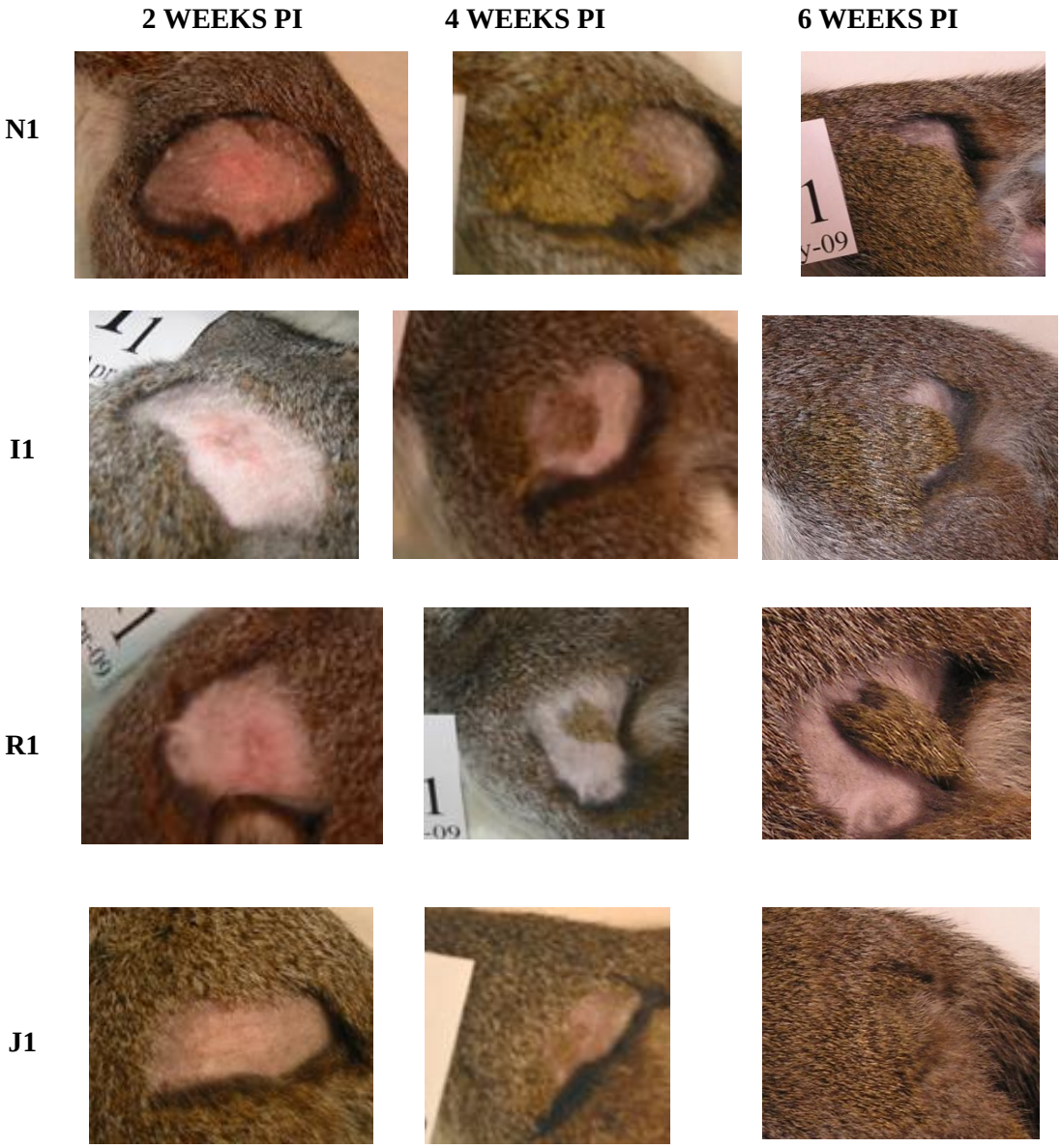


Fig 5.12 Skin lesions at the scarification site, long term infection.

Four representative infected squirrels illustrating skin healing at the scarification site between weeks two to six post infection.

Table 5.10 Long term infection biometric data and difference in body weight (%) at the end of experiment.

Squirrel	Gender	Crown-rump (CRL) (mm)	Shin length (SL) (mm)	Crown-rump/shin length	Body weight(g) end point experiment	Index	Body mass fluctuation (%)
K1	F	210	80	2.6	522.7	199.1	-6.16
L1	F	215	82	2.6	540.0	206.0	-9.24
P1	M	185	80	2.3	513.3	222.0	-1.85
O1	M	210	85	2.5	572.0	231.5	-4.83
J1	M	205	75	2.7	545.0	199.4	-0.37
R1	M	210	80	2.6	582.0	221.7	-3.59
G1	F	205	70	2.9	563.8	192.5	+2.14
H1	M	185	70	2.6	566.3	214.3	-8.44
I1	M	205	75	2.7	559.0	204.5	-4.61
N1	M	197	77	2.6	601.1	234.9	-4.59
Q1	M	192	68	2.8	589.2	208.7	-0.47

Individual body weight (g) at the end of the experiment and body weight's fluctuations expressed as a percentage change (start weight compared to weight at end of experiment) over the course of the long term experiment. The index allows a comparison of different individuals and has been corrected for body size (body weight (g)/(CRL/SL)).

Mean group body weight and body size (crown-rump and shin length, see **Table 5.10**) showed no noticeable difference between the individuals used in the short and long term experiment (**Table 5.8**). However, in contrast to the short term experiment, 10 out of the 11 animals showed a slight decrease (%) in body weigh over the course of the experiment (range 0.37-9.24%) suggesting possible long term stress as a result of captivity.

Table 5.11 Liver and spleen measurements in the long term infection.

squirrel	gender	liver weight (g)	spleen weight (g)	spleen length (mm)	spleen width (mm)
K1	F	13.6	1.5	57	16
L1	F	18.1	1.6	46	14
P1	M	13.8	1.2	40	14
O1	M	19.0	1.5	55	17
J1	M	16.4	1.9	48	15
R1	M	20.6	1.3	58	14
G1	**F**	**14.3**	**1.2**	**40**	**15**
H1	M	17.4	1.5	45	17
I1	**M**	**17.2**	**1.7**	**45**	**17**
N1	**M**	**17.8**	**1.5**	**47**	**17**
Q1	**M**	**14.0**	**2.1**	**47**	**15**
MEAN		**16.6**	**1.5**	**48.0**	**15.5**

Liver and spleen weights and spleen size (length and width) for 11 squirrels in the long term infection experiment. SQPV ELISA positive squirrels are indicated in bold.

Again there was no correlation (Pearson's correlation: r=0.3898, N=11, p=0.236) between ELISA positive status of individuals (**Table 5.7** and **5.11**) and their relative spleen weight. In addition, there were no macroscopic pathological changes evident in these samples.

5.7 Detection of viral DNA in samples collected at necropsy

For the purpose of detecting SQPV DNA and to enable the quantification of relative viral load in different tissue types, a qPCR assay was carried out on samples collected during the post mortem examination (more details on procedures given in **Chapter 2**). Samples from the short and long term infection are combined for the purposes of presentation.

5.7.1 Tissue samples

The qPCR results from the different tissue samples, blood, swabs, urine and faeces collected during post mortem examination of the squirrels are presented in **Tables 5.12 -A-**, **5.12-B-** and **5.12-C-**. Samples were collected from the lymphoreticular, integumentary, digestive, genitourinary, respiratory and cardiovascular system. Within the integumentary system skin samples were collected not only at the site of scarification, but also from distant sites.

Due to the large number of samples collected post-mortem, not all were analysed by qPCR, but instead were prioritised both by time (DPI) and as informed by the analyses in **Chapters 3** and **4**. However, for squirrels in the two groups euthanized eight and 16 days post infection, all the samples collected were analyzed. The mock infected squirrel (L2) was included with those euthanized eight DPI to compare with the infected squirrels.

Table 5.12-A- qPCR results expressed as mean C_p value for different squirrel tissues.

Days post infection	2DPI			4 DPI			6DPI			8 DPI			
Squirrel / Tissue	C2	O2	V1	B1	D1	H2	A1	C1	G2	D2	E2	J2	L2"
Lymphoreticular system													
SM LN	/	+	/	/	/	/	/	/	/	/	/	/	/
PALATINE TONSIL										/	/	/	
SPLEEN	/	/	/	/	/	/	/	/	/	/	/	/	/
THYMUS										/	/	/	/
POPL. LYMPH NODE R.	/	+	37***						/	/	/	/	/
POPL. LYMPH NODE L.	/	/	/							/	/	/	/
OTHER LYMPH NODE									/				
Integumentary system													
EYELID SKIN	/	/	+	/	+	+	+	/	+	/	/	/	/
HARDERIAN GLAND										/	/	/	/
POST. DIGITAL SKIN		+							+	/	+	+	/
ANTER. DIGITAL SKIN	/	+	/	/	+	/	/	/	+	/	/	/	/
EAR SKIN	/	+	/	/	/	/	/	/	/	/	/	/	/
NASAL SKIN										/	+	/	/
MOUTH SKIN									/	/	/	/	/
MUZZLE SKIN	/	+	/	+	+	/	/	/	/	/	/	/	/
SCARIFICATION SKIN	25	24	21	22	24	21	22	22	22	26	30	32	
MOCK SKIN	+	+	+	+	37**	+	/	+	+	/	+	/	/
AXILLARY SKIN	/	/	/	/	+	/	/	/	+	/	/	/	/
SCAB							33						
SCENT GLAND	/	/	/	/	/	/	/	/	/	/	/	/	/
Digestive system													
PAROTID	/	/	/	/	/	/	/	/	/	/	/	/	/
SM SG					+		/	+		/	/	/	/
LIVER	/	/	/	/	/	/	/	/	/	/	/	/	/
TONGUE										/	/	/	/
STOMACH										/	/	/	/
SMALL INTESTINE										/	/	/	/
CAECUM										/	/	/	/
LARGE INTESTINE										/	/	/	/
RECTUM										/	/	/	/
Other internal organs													
LUNG R/A										/	/	/	/
KIDNEY	/	/	/	/	/	/	/	/	/	/	/	/	/
GONADS			/			/				/	Ns	/	/
HEART										/	/	/	/
Swabs													
OCULAR SWAB	/	+	/	/	+	/	/	/	/	+	/	+	/
SCAR. SKIN SWAB	33	36	32							+	33	37	/
ORAL SWAB	+		35	/	36	/	+	+	+	+	+	+	/
SWAB OF NEST LID				+	+	+	+	+	37				
Others													
BLOOD IN EDTA	/	/	/	/	/	/	/	/	/	/	/	/	/
URINE		/											
FAECES	/	/	/	Ns	/	/	/	/	/	/	/	/	/

Table 5.12-B- qPCR results expressed as mean C_p value for different squirrels tissues.

Days post infection / Squirrel / Tissue	10 DPI			12 DPI			14DPI			16DPI		
	E1	F1	I2	B2	I3	K2	G3	H3	P2	F2	M2	N2
Lymphoreticular system												
SM LN	/	/	/	/	/	/	/	/	/	/	/	/
PALATINE TONSIL										/	/	/
SPLEEN	/	/	/	/	/	/	/	/	/	/	/	/
THYMUS										/	/	/
POPL. LYMPH NODE R.	33	/	37*	/	/	+	/	/	/	/	/	/
POPL. LYMPH NODE L.	/	/	/	/	/	/	/	/	/	/	/	+
OTHER LYMPH NODE	/											
Integumentary system												
EYELID SKIN	+	/	+	/	/	/	/	/	/	/	/	+
HARDERIAN GLAND										/	/	/
POST. DIGITAL SKIN	+		/				+		+	+	+	35
ANTER. DIGITAL SKIN	/	+	+	+	+	/	+	/	/	/	+	35
EAR SKIN	/	/	+	/	/	/	/	/	/	/	/	+
NASAL SKIN										/	+	36
MOUTH SKIN							/	/		/	+	+
MUZZLE SKIN	+	+	/	+	/	/	/	/	/	/	/	35
SCARIFICATION SKIN	33	34	33	32	33	33	34	33	32	34	35	32
MOCK SKIN	+	37**	/	/	/	+	/	/	/	/	37***	34
AXILLARY SKIN	/	+	/	/	/	/	/	/	+	/	/	+
SCENT GLAND	/	/	/	/	/	/	/	/	/	/	/	+
Digestive system												
PAROTID	/	/	/	/	/	/	/	/	/	/	/	/
SM SG										/	/	/
LIVER	/	/	/	/	/	/	/	/	/	/	/	/
TONGUE										/	/	/
STOMACH										/	/	+
SMALL INTESTINE										/	/	/
CAECUM										/	/	/
LARGE INTESTINE										/	/	/
RECTUM										/	/	/
Other internal organs												
LUNG R/A										/	/	/
KIDNEY	/	/	/	/	/	/	/	/	/	/	/	/
GONADS		/								/	/	/
HEART										/	/	/
swabs												
OCULAR SWAB	+	/	/	+	/	/	+	/	/	/	/	37
SCAR. SKIN SWAB				+	36	37	+	35	35	33	33	32
ORAL SWAB	+	/	+	/	37	/	/	/	/	+	/	37
SWAB OF NEST LID	+	+	+									
Others												
BLOOD IN EDTA	/	/	/	/	/	/	/	/	/	/	/	/
FAECES	/											

Table 5.12-C- qPCR results expressed as mean C_p value for different squirrels tissues.

Days post infection	18 DPI			21 DPI					21 DPI second challenge			28 DPI	42 DPI	
Squirrel / tissue	T1	U1	A2	G1	H1	K1	L1	P1	I1	N1	Q1	O1	J1	R1
Lymphatic system														
SM LN	/	/	/	/	/	/	/	/	/	/	/	/	/	/
PALATINE TONSIL				/										
SPLEEN	/	/	/	/	/	/	/	/	/	/	/	/	/	/
THYMUS					/									
POPL. LYMPH NODE R.	/	/	/											
POPL. LYMPH NODE L.	/	/	/											
OTHER LYMPH NODE								/	/					
Integumentary system														
EYELID SKIN	/	/	/	/	/	/	/	+	+	/	+	+	/	+
HARDERIAN GLAND							/					/		
POST. DIGITAL SKIN	/		+		+					31				
ANTER. DIGITAL SKIN	/	/	+	+	+	/	/	35	+	/	+	+	/	37
EAR SKIN	/	/	/	/	/	/	/	/	/	/	+	/	/	/
MOUTH SKIN				/										
MUZZLE SKIN	/	/	/			/	/	/				/		
SCARIFICATION SKIN	34	33	35	/	34	37	/	+	30	33	37	+	/	+
MOCK SKIN	/	/	+	/	/				/	+	/			
AXILLIA SKIN	/	/	+	/	/	/	/	/	+	/	/	+	/	/
SCAB										+		+		
SCENT GLAND	/	/	/	/	/	/	/	/	/	/	/	+	/	/
Digestive system														
PAROTID	/	/	/	/	/	/	/	/	/	/	/	/	/	/
LIVER	/	/	/	/	/	/	/	/	/	/	/	/	/	/
STOMACH				/										
Other internal organs														
HEART										/				
KIDNEY	/	/	/	/	/	/	/	/	/	/	/	/	/	/
swabs														
OCULAR SWAB	/	/	+	+	+		/	/	+	/	/	/	+	
SCAR. SKIN SWAB	+	37	37											
ORAL SWAB	/	/	/	+	+	/		/	37	+	35	37	/	/
Others														
BLOOD EDTA	/	/	/											

Tables –**A-**, -**B-** and –**C-** show qPCR results from squirrel tissues samples. The Cp scores are expressed as a mean Cp value for four replicates of the same sample. In the cases where not all four replicates tested positive, each positive replicate is indicated by '*'. Cp scores above 37, the detection limit, with one or more positive replicates out of four are represented as '+'; negative results as '/' and a blank space where no sample was analyzed. L2 is the uninfected squirrel.

SM LN: submandibular lymph node; POPL. LYMPH NODE R: Right popliteal lymph node; POPL. LYMPH NODE L: Left popliteal lymph node; OTHER LYMPH NODES: additional (out of protocol) lymph nodes collected during post mortem; POST. DIGIT. SKIN: portion of plantar hind paw skin; ANTER. DIGIT. SKIN: portion of plantar fore paw skin. SCARIFICATION SKIN: SQPV skin from scarification site; MOCK SKIN: mock scarified skin; SM SG: submandibular salivary gland; LUNG R/A: lung, right apical lobe; SCARIF. SWAB: swab taken from the SQPV skin scarification site surface.

Consistent positive results, with the largest amount of viral DNA detected were obtained from samples (and swabs) taken from the surface of the scarified skin. Generally, positive qPCR results were associated with the integumentary system and in particular with digital, eyelid, mock infected and axillary skin tissue samples, but swabs of the oral mucosa and from the ocular surface were also positive in some animals.

Figure 5.13 gives an overview of all the results from the site of scarification for each animal illustrating that virus DNA could be detected successfully from the majority of experimental infected animals. Samples from the individuals that tested negative are indicated by blank columns. Virus was successfully detected in 35 out of 38 animals. The control animal which was not infected (L2) is excluded from the figure.

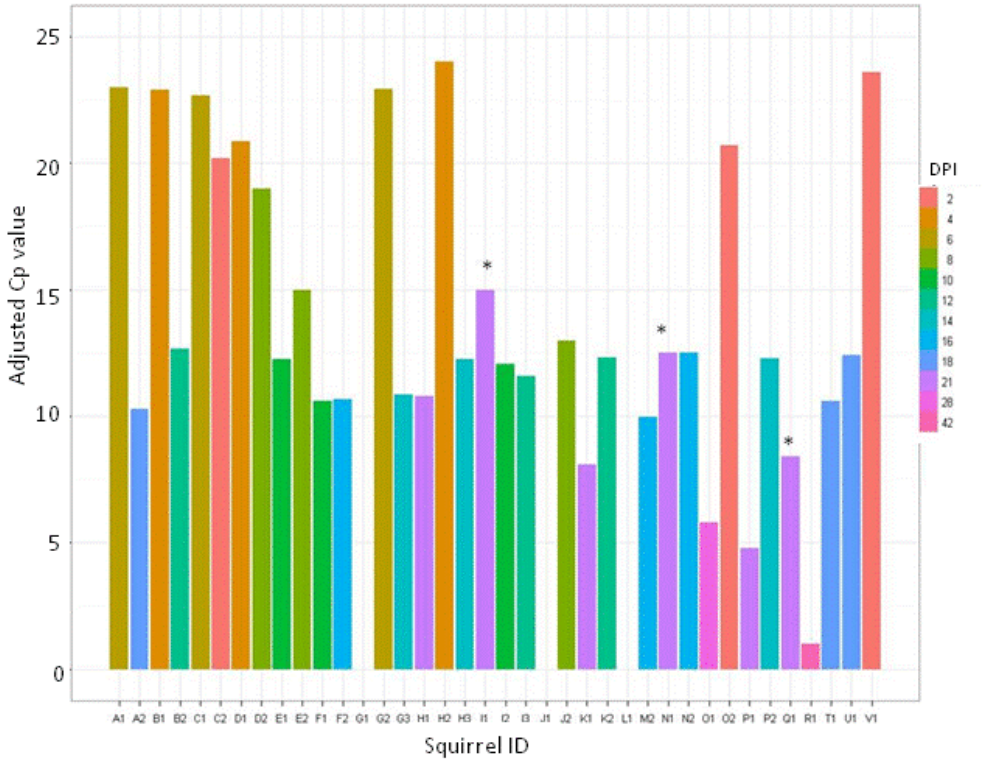


Figure 5.13 qPCR adjusted C_p values for each squirrel from SQPV scarified skin, represented as bars.

Individual C_p values from SQPV scarified skin, with the highest concentration of virus proportional to the bar length. On the y-axis, the adjusted C_p value is given; this is the actual C_p value from each assay subtracted from 45 which represents the limit of the thermal cycles used for the assay. On the x-axis is the squirrel ID. The blank columns represent qPCR negative results. Purple columns with asterisks indicate squirrels that were re-challenged and their tissues samples were collected 21 days after the second challenge. The uninfected squirrel L2 is omitted from the graph.

Figure 5.14 illustrates the relative quantities of viral DNA detected in SQPV scarified skin at each of the examined time points post infection. As might have been predicted from the mild scabby lesions that developed on the scarified skin (§**5.6.1**), comparatively high concentrations of viral DNA were detected between days two to eight post infection with the peak from four to six days post infection. Thereafter, there is a lower, but consistent, amount of virus DNA detected up to 21 DPI. The box plot clearly shows that within a group the variation increased at and beyond 21 DPI, with two squirrels testing negative at 21 DPI and one out of two at day 42 post infection.

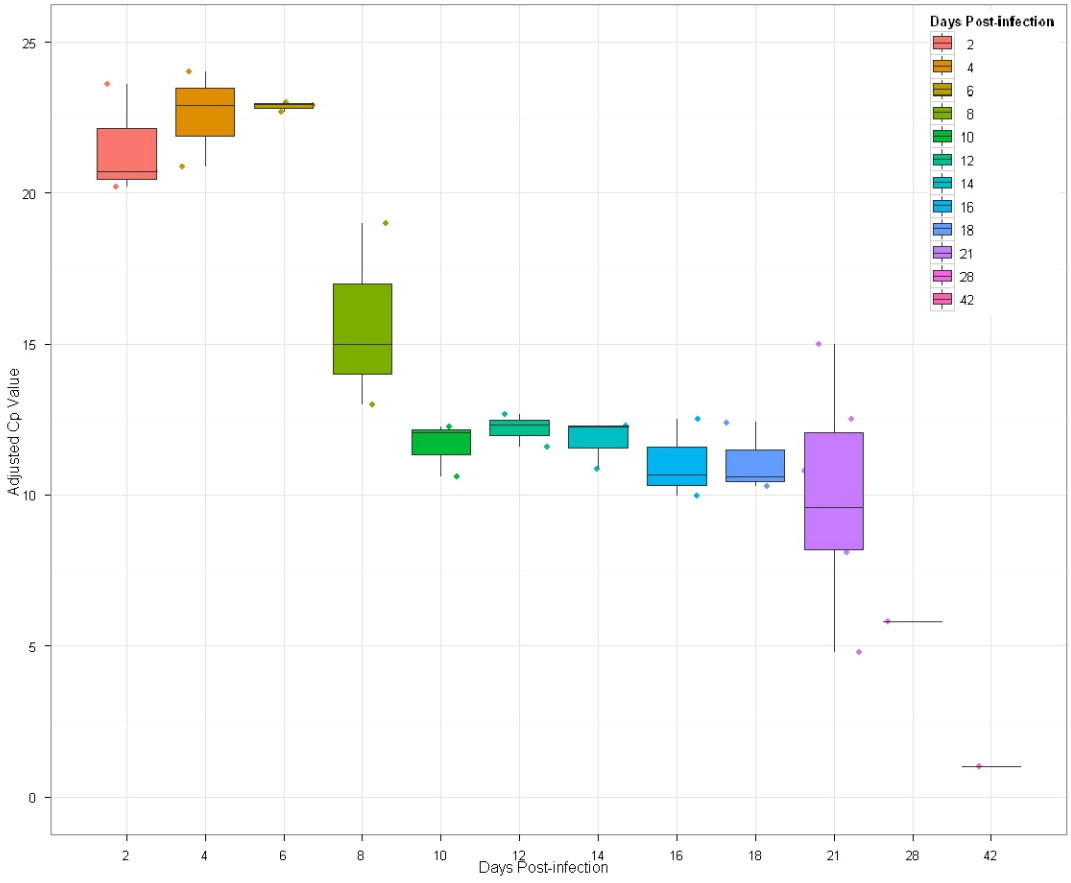


Figure 5.14 Box plots for qPCR adjusted values from scarified skin samples plotted against DPI.

The medians, quartiles, minimum and maximum values are shown. On the y-axis, the adjusted Cp value is the actual Cp value subtracted from 45 which represents the limit of the thermal cycles used for the assay. The x-axis indicates DPI.

Using the standard curve presented earlier (§**5.2.1**), the mean amount of virus DNA detected (genome equivalent/µg of total DNA isolated) in the scarified skin samples was calculated for each group. In the period from two to six DPI, approximately 1.5 x 10^7 genome equivalents were detected per microgram of total DNA isolated from the tissue sample. From eight DPI the virus load decreased markedly with an estimated value of 1.5 x 10^5 genome equivalents /µg of total DNA isolated, decreasing to 1.5 x 10^4 at 10 DPI and 1.5 x 10^3 genome equivalents /µg of total DNA isolated at 16 DPI. At 18 DPI the value corresponded to between 1.5 x 10^3 and 1.5 x 10^2 genome equivalents /µg of total DNA isolated.

At 21 DPI two animals tested qPCR negative while the other three were positive with a mean Cp score at the limit of the qPCR detection (approx. 1.5 x 10^2 genome equivalents/µg of total DNA isolated). In contrast, test results for the group of three animals that had been challenged a second time still showed a mean virus load estimated to be 1.5 x 10^3 genome equivalents/µg of total DNA isolated at 21 DPI. Only one animal was tested at 28 DPI giving a Cp score of 39 corresponding to <30 genomes equivalent per reaction.

The last group tested at 42 DPI, comprised one negative and one positive result. However, the mean Cp value was 44 for only three positive replicates out of four. This may indicate that viral DNA was present but at very low concentrations (<3 genome equivalents in the reaction mix).

The decrease in Cp score (increase in viral DNA) between two DPI (mean±1SD = 23.3±2.08), four DPI (22.3±1.53) and six DPI (22±0.0) together with the observed decrease in variation (standard deviation) could suggests that the virus was replicating at the scarification site up to six DPI.

For the swab samples, the ones taken with a gentle scrape from the SQPV infected skin area gave moderately positive results within the group of 18 squirrels tested. Squirrel L2 (non infected) gave negative results. These 18 positive results corresponded to three animals at two DPI, three at eight DPI, three at 12 DPI, three at 14 DPI, three at 16 DPI, three at 18 days post infection.

Of 35 swabs taken from the oral mucosa, 20 tested positive (approx. 71%). Of these, seven were estimated to contain between 1.5 x 10^2 and 1.5 x 10^3 genome

equivalents/µg of total DNA isolated from each swab, and the remaining 13 positive samples had a smaller amount of viral DNA. However, there was no clear temporal pattern within the seven samples that contained more viral DNA.

Ocular swabs gave similar results. However, the amount of virus DNA detected was always lower than the oral swabs and the only consistent positive result, having 4 positive replicates out of four and with a Cp value still within the range of the standard curve, was squirrel N2. In all 37 ocular swabs, 13 tested weakly positive. There was no specific temporal pattern, with virus DNA being detected from two up to 42 DPI.

Seven out of 88 lymph nodes tested positive for SQPV DNA. Positive results were obtained from the popliteal lymph nodes draining the scarification sites two DPI, 10 DPI and 12 DPI and from the contra-lateral popliteal lymph node from one squirrel (N2) at 16 DPI. The submandibular lymph nodes were analyzed from all squirrels, but were negative with the exception of one animal (O2) which had a positive result (two DPI). The qPCR results however indicated a much lower level of virus DNA than was found in the popliteal lymph nodes. Given the moderate enlargement of the brachial lymph nodes observed in some squirrels (G2, E1, P1 and I1) during the post mortem examination (see **Figure 5.11** squirrel E1), these were also collected for qPCR analysis but none of them tested positive.

Some of the squirrels exhibited mild scabs in the groin region and extending to the distal part of the hind limbs prior to the start of the experiment. It was thought these scabs were consistent with ringworm infection, although cultures were not undertaken for the isolation of a specific pathogen. These scabs were however collected at necropsy even though they were not thought to be associated with SQPV infection. Nevertheless, in one squirrel (O1, euthanized 28 DPI) the scab from the medial aspect of the hind limb was positive even though the animal had been scarified on the lateral side of the thigh. The amount of virus DNA detected was relatively low (Cp =40) with two positive replicates out of four, similar to another squirrel scab sample (N1; 21 days post second challenge) which gave a value of Cp =38 but with three positive replicates out of four. For N1 the sample was collected from the same side of SQPV scarified limb. -For comparison, for another animal (A1,

six DPI) a scab sample was collected from the edge of the scarification site and as expected, the virus load was relatively high (Cp 33) showing a consistent amount of virus DNA.

Squirrel N2 (euthanized 16 DPI) was of particular interest. Samples analyzed from the scarified skin and a swab of the scarified skin surface, were both shown to contain approximately the same amount of viral DNA, calculated to be between 1.5 x 10^4 and 1.5 x 10^5 genome equivalents/µg of total DNA isolated. Indeed this value was the highest among the group of squirrels sacrificed on the same day, where virus load for scarified skin was between 1.5 x 10^3 and 1.5 x 10^2 genome equivalents/µg of total DNA isolated. Moreover, the mock scarified skin for that animal was also positive with a Cp score =32 comparable to the mean Cp value obtained from SQPV scarified skin samples of squirrels belonging to groups 10, 12, 14 and 16 DPI. Swabs and samples from the integumentary system (except for the harderian gland which was negative) were also all positive for squirrel N2. In addition, it was the only individual for which stomach and scent gland samples were positive. However, for these last two samples the result was not very consistent with only one positive replicate out of four and a Cp value corresponding to a virus load lower than 30 genome equivalent in the reaction mix.

5.7.2 Blood and Faecal samples

No viral DNA was detected in blood samples from any of the 39 squirrels at any time throughout the experiment. This suggested that a viraemia was not part of the normal clinical progression of disease caused by squirrelpox virus, at least when experimentally induced. Other qPCR results also appeared to be consistent with an absence of viraemia in that none of the internal organ samples such as kidney, spleen, liver or lung tested positive for viral DNA.

Faecal samples were collected from infected animals at various time points as well as from the one uninfected squirrel. Analyses were focused on samples taken during the two week period post infection. Due to the lack of evidence for viraemia and the absence of viral DNA in the kidney samples examined, only one urine sample was analyzed. None of the faecal samples, or the urine sample, contained any detectable viral DNA.

5.7.3 Swabs of solid surfaces

Swabs were taken from the top external surface (lid) of nine nest boxes, four weeks after the end of the experiment, in order to test for potential environmental contamination with the virus. Although the amount of DNA detected was low, all nine nest-box lids tested positive for viral DNA confirming the possibility of viral contamination at nest sites.

5.8 Histopathology

Histological examination of the skin at the site of scarification was undertaken at each sampling point during the course of the experiment. The results showed that at each time point, the lesions were confined in the scarified skin and in all scarified squirrels, lesions occurred predominantly in the epidermis and dermis corresponding to the site of the SQPV challenged skin surface. Other tissues examined did not show any sign of pathology as predicted from post mortem examination.

5.8.1 Short term infection

Histopathological examination of skin lesions from SQPV scarified squirrels generally showed a noticeable difference compared with the contra-lateral mock infected skin area. These differences become more accentuated after four to six days post infection. Between two and four DPI the presence of extensive sero-cellular crusting was detected in both skin samples (SQPV infected and mock infected), but the underlying erosion had not breeched the epidermal basal membrane. Moreover, the sero-cellular crust persisted in the SQPV infected squirrels for much longer compared to the mock infected squirrels (see **Figure 5.15**–**B-**).

The difference between SQPV infected and mock challenge could been seen as early as four DPI (**Figure 5.15**–**A-**) when a mild inflammatory infiltrate was present in all SQPV scarified skin samples but was either absent in all the mock scarified samples or if present, the degree of inflammation was lower. Usually mock scarified skin was fully re-epithelialised within eight days and the dermis underlying the epidermal lesions had a very mild inflammatory infiltrate in some samples, while in contrast in

the SQPV scarified the inflammation became more evident after few days post infection.

Generally between two and 12 DPI, the epidermis corresponding to the site of SQPV scarification was moderately thickened with hyperplasia (acanthosis) at the periphery of the lesions and moderate acantholysis. Epidermal necrosis was characterized by pyknosis and karyorrhexis with a mild inflammatory infiltrate and spongiosis.

A diffuse neutrophilic inflammatory infiltrate, generally underlying the superficial and mid dermis, was also present. Initially the dermis underlying the scarified skin had a mild to moderate infiltrate of mononuclear cells that were predominantly lymphocytes with some plasma cells. The inflammatory infiltrate in the dermis also contained some neutrophils (e.g. **Figure 5.15**–**B-**, slide **5**). With the progression of time, the inflammatory infiltrate in the dermis was more consistent, with a predominance of mononuclear cells (lymphocytes, macrophages and plasma cells). For some samples, exocytosis in the upper epidermis was also present as a mild mononuclear infiltrate (**Figure 5.15**–**B-**, slide **7**).

Between two and four DPI, the microscopic appearance of the skin lesions in SQPV scarified squirrels had the characteristic of a superficial erythematous dermatatitis with mild focal hyperplasia. In some cases these features extended much deeper with the mixed inflammatory infiltrate reaching, where extending most deeply, the panniculus. Normally the epidermis showed a mild suprabasilar acantholysis, spongiosis and varying degrees of ballooning degeneration (e.g. **Figure 5.15**–**A-**, slide **3**) with some pyknotic keratinocytes.

After four DPI histopathological examination showed generally a more marked hyperkeratosis, spongiosis and mild acanthosis, hydropic degeneration of small numbers of keratinocytes and marked dermal inflammation. These finding become progressively more evident for the majority of samples with skin lesions being observed after six DPI and becoming less prominent after 16 or 18 DPI (data not shown).

Between six and eighth DPI the dermal inflammation progressed and the major changes were observed in the epidermis of infected animals. The epidermis was thickened with focal hyperplasia, spongiosis of keratinocytes become more

prominent and characteristic elongated rete ridges and focally extensive hyperplastic nodular dermatitis was characteristic for some samples (**Figure 5.15**–**B-** slide **5**).

From 12 to 16 days post infection histopathological examination revealed a continuous recruitment of inflammatory cells underlying areas of epidermal hyperplasia. The inflammatory infiltrate was mainly composed of mononuclear cells such as lymphocytes, but a few eosinophilss were also recognized in the dermis. The overlying epidermis continued to show several types of histological change such as hyperplasia and degeneration of keratinocytes. These changes included vacuolization and necrosis of basal keratinocytes that appeared to be associated with infiltration and activity of lymphocytes resulting in some necrotic ketatinocytes (colloid bodies) in the epidermis (**Figure 5.15**–**C**- slide **8**).

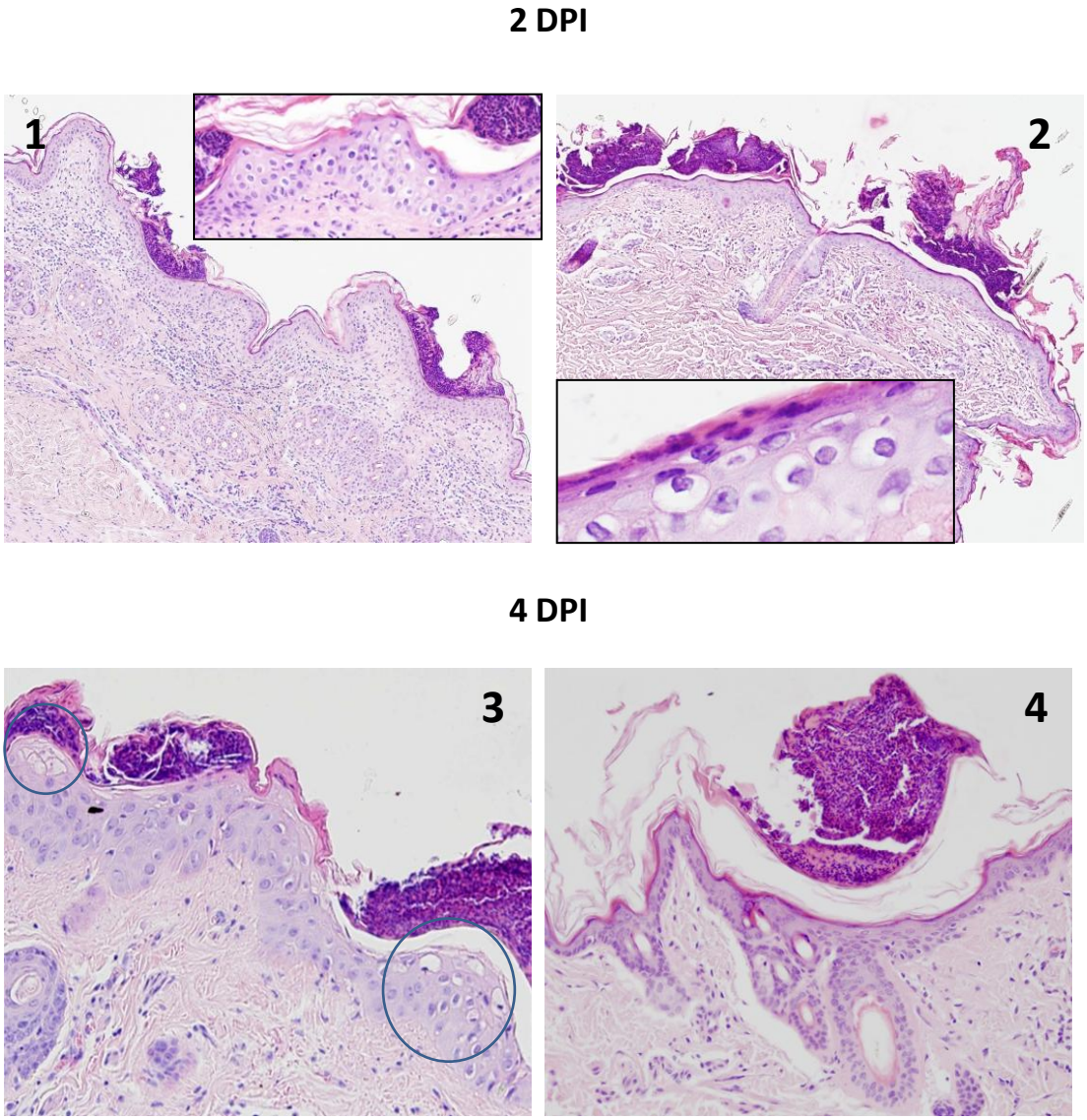


Figure 5.15 -A- Section of primary lesion from SQPV scarified and mock scarified skin at two and four DPI. Haematoxylin and eosin staining.

SQPV scarified skin is on the left side and mock scarified skin is on the right (contralateral thigh). **1)** Squirrel O2: Superficial and deep dermatitis with mild focal epidermal hyperplasia with sero-cellular crust formation. Mixed inflammatory infiltrate extending into the underlying dermis and reaching the panniculus. Original magnification x 40. Inset: high power view of the epidermis showing hydropic degeneration of keratinocytes. Original magnification x 100. **2)** Squirrel O2: mock scarified skin. Presence of sero-cellular crust formation and very mild inflammatory infiltrate in the underlying superficial dermis. Original magnification x 40. Inset: detail of epidermis showing hydropic degeneration of keratinocytes. Moderate parakeratotic hyperkeratosis. Original magnification x 400. **3)** Squirrel B1: Superficial dermatitis and presence of sero-cellular crust above the scarification site. The epidermis is moderately thickened with mild hyperplasia. Circled: ballooning degeneration of keratinocytes with pyknotic nuclei. Original magnification x 100 **4)** Mock scarified skin from squirrel B1: sero-cellular crust above the epidermis. Normal appearance of the skin. Original magnification x 100

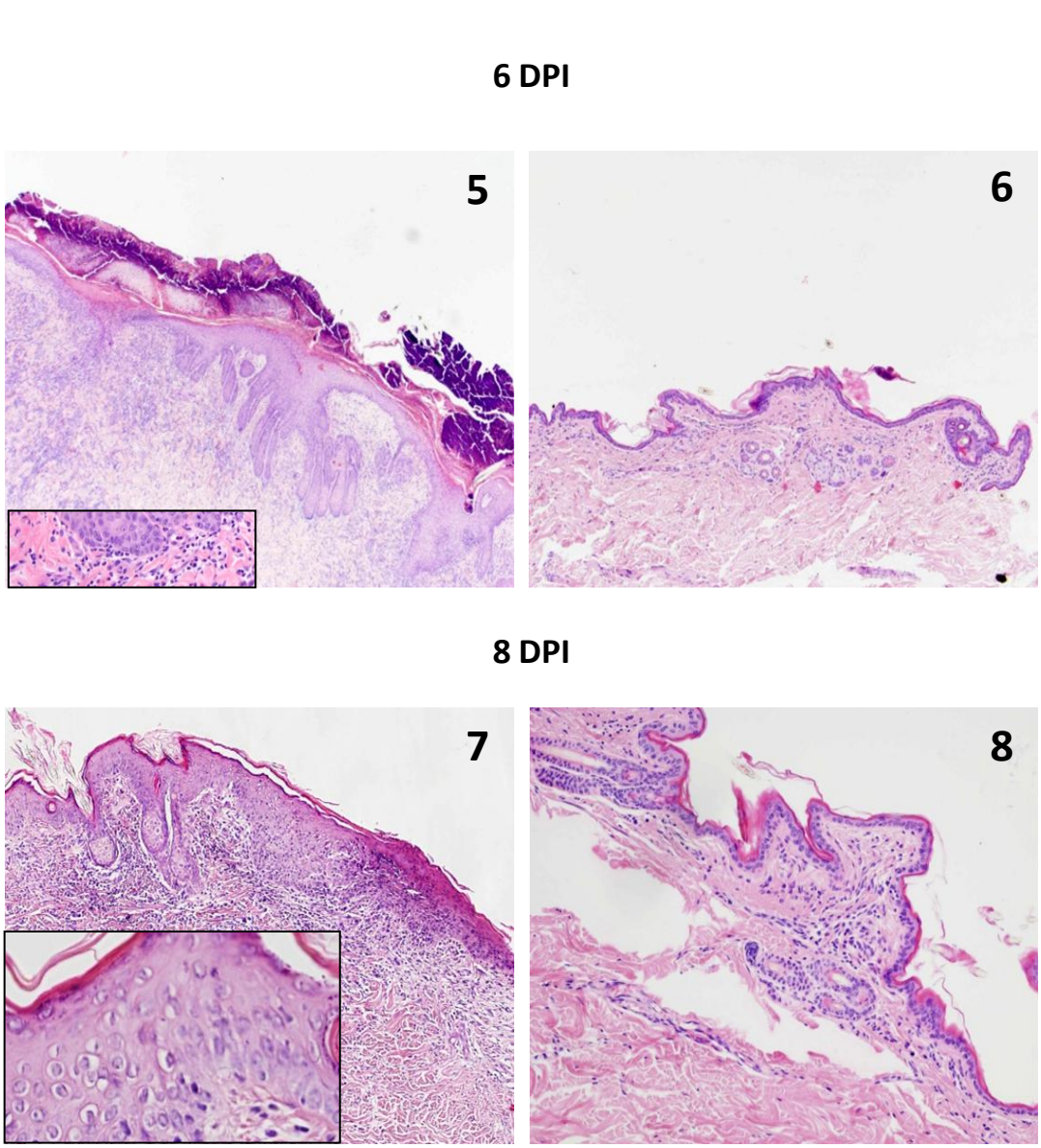


Figure 5.15 -B- Section of primary lesion from SQPV scarified and mock scarified skin at six and eight DPI. Haematoxylin and eosin staining.

SQPV scarified skin is on the left side and mock scarified skin is on the right (contralateral thigh). **5)** Squirrel A1: Elongated rete ridges characteristic of focally extensive hyperplastic nodular dermatitis with extensive sero-cellular crust formation. Diffuse neutrophilic inflammatory infiltrate in the underlying superficial and mid dermis. Original magnification x 20. inset: detail of inflamatory infiltarte in the superficial derma. Original magnification x 100. **6)** squirrel A1: mock scarified skin. Mild focal orthokeratotic hyperkeratosis. Very mild inflammatory infiltrate in the underlying superficial dermis. Original magnification x 40. **7)** Squirrel E2: Focal interface dermatitis with extensive mixed inflammatory infiltrate and overlying sero-cellular crust at the scarification site. The epidermis is moderately thickened with focal hyperplasia. Moderate parakeratotic hyperkeratosis. Original magnification x 40. Inset: detail of epidermis showing hydropic degeneration of keratinocytes and a few lymphocytes in the deeper epidermis. Original magnification x 200. **8)** Mock scarified skin from uninfected squirrel L2:. Normal appearance of the skin. Very mild inflammatory infiltrate in the underlying the superficial dermis. Original magnification x 100.

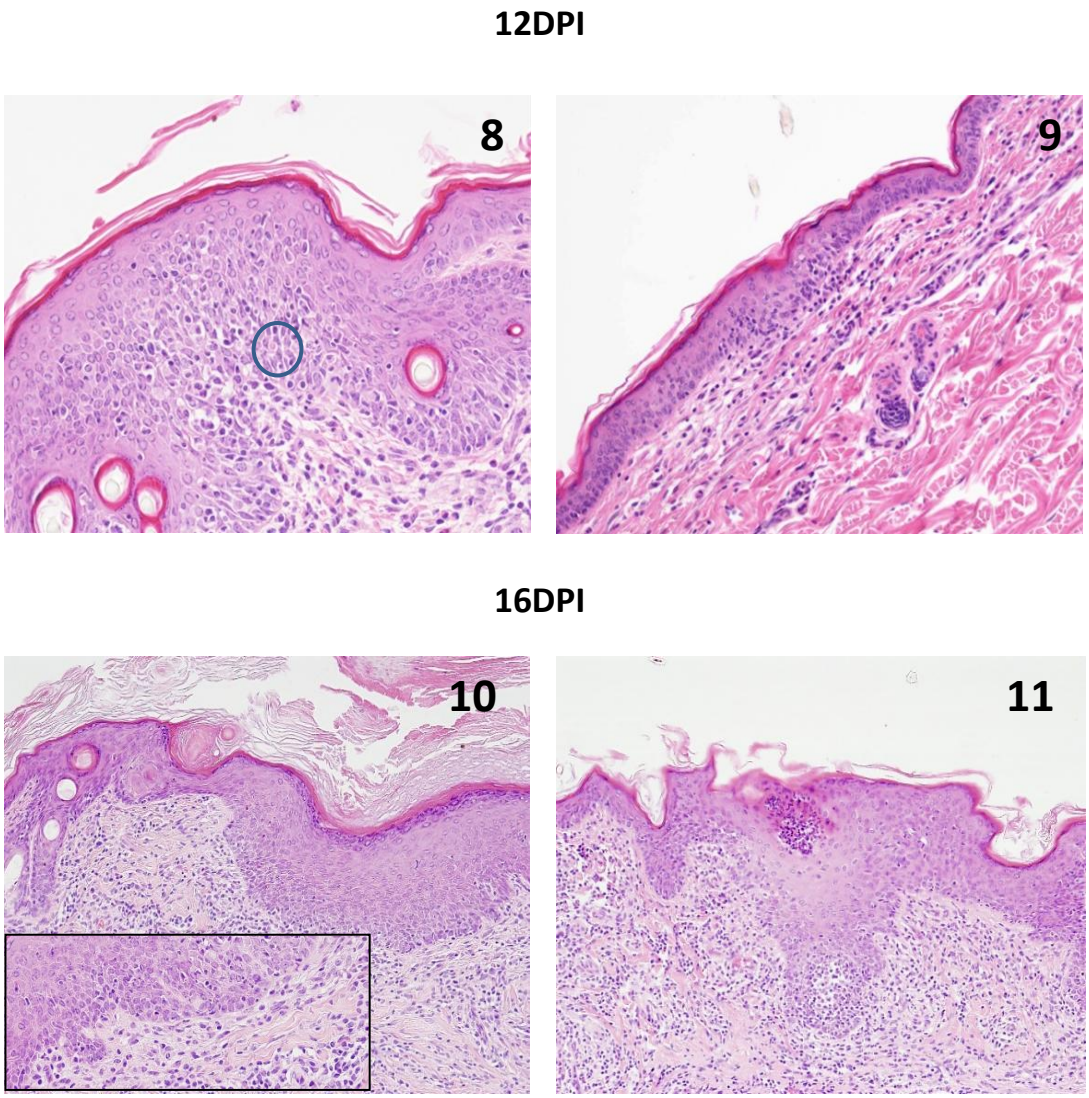


Figure 5.15 -C- Section of primary lesion from SQPV scarified and mock scarified skin at 12 and 16 DPI. Haematoxylin and eosin staining.

SQPV scarified skin is on the left side and mock scarified skin on the right (contralateral thigh). **8)** Squirrel I3: prominent spongiosis and hyperplasia with mild orthokeratotic hyperkeratosis and lymphocyte exocytosis. The underlying dermis contains an inflammatory infiltrate mainly composed of lymphocytes and a few plasma cells. The figure also shows a few keratinocytes with intracellular oedema. Circled in blue is a colloid body probably resulting from keratinocyte degeneration. Original magnification x 200. **9)** Squirrel I3: mock scarified skin. Focally mild epidermal hyperplasia. Mild inflammatory infiltrate in the superficial dermis mainly localised deep to the hyperplastic epidermis. Original magnification x 100. **10)** squirrel N2: moderate hyperplasia and spongiosis in the stratum spinosum. Mixed inflammatory infiltrate with lympocytes also extend into the upper epidermis, with a few neutrophils and plasma cells. The epidermis shows moderate orthokeratotic hyperkeratosis. Original magnification x 100. Inset: high power view of the epidermis-dermis junction showing in more detail the mixed inflamatory infiltrate. Original magnification x 200. **11)** Squirrel N2: mock scarified skin. Note the similarity with the SQPV scarified skin lesion. Moderate epidermal hyperplasia and a moderate dermal inflammatory infiltrate with lymphocytes and a small number of neutrophils. There is a microabscess in the superficial epidermis. Original magnification x 100.

5.8.2 Long term infection

For the long term infection, histopathological examination was carried out between 21 and 42 DPI. Unlike the protocol for the short term infection some of the animals (I1, N1, Q1) were challenged with SQPV a second time in the same skin area 70 days after the first challenge and euthanized 21 days later.

Generally, skin lesions from SQPV challenged squirrels were less well defined compared to the short term experiment. However, 21 DPI (first challenge) a moderate hyperplasia with a mild superficial inflammatory infiltrate was still present in some animals (**Figure 5.16** slide **1**). In contrast the mock infected skin only showed some mild superficial dermal inflammation at 21 DPI with later samples being normal.

Of particular interest was the difference between dermal histopathology from animals challenged only once and animals re-challenged a second time. Dermal inflammation and epidermal hyperplasia with hydropic degeneration of some keratinocytes was much more pronounced in animals challenged only once compared to those that were re-challenged (e.g. compare **Figure 5.16** slides **1** and **5**)

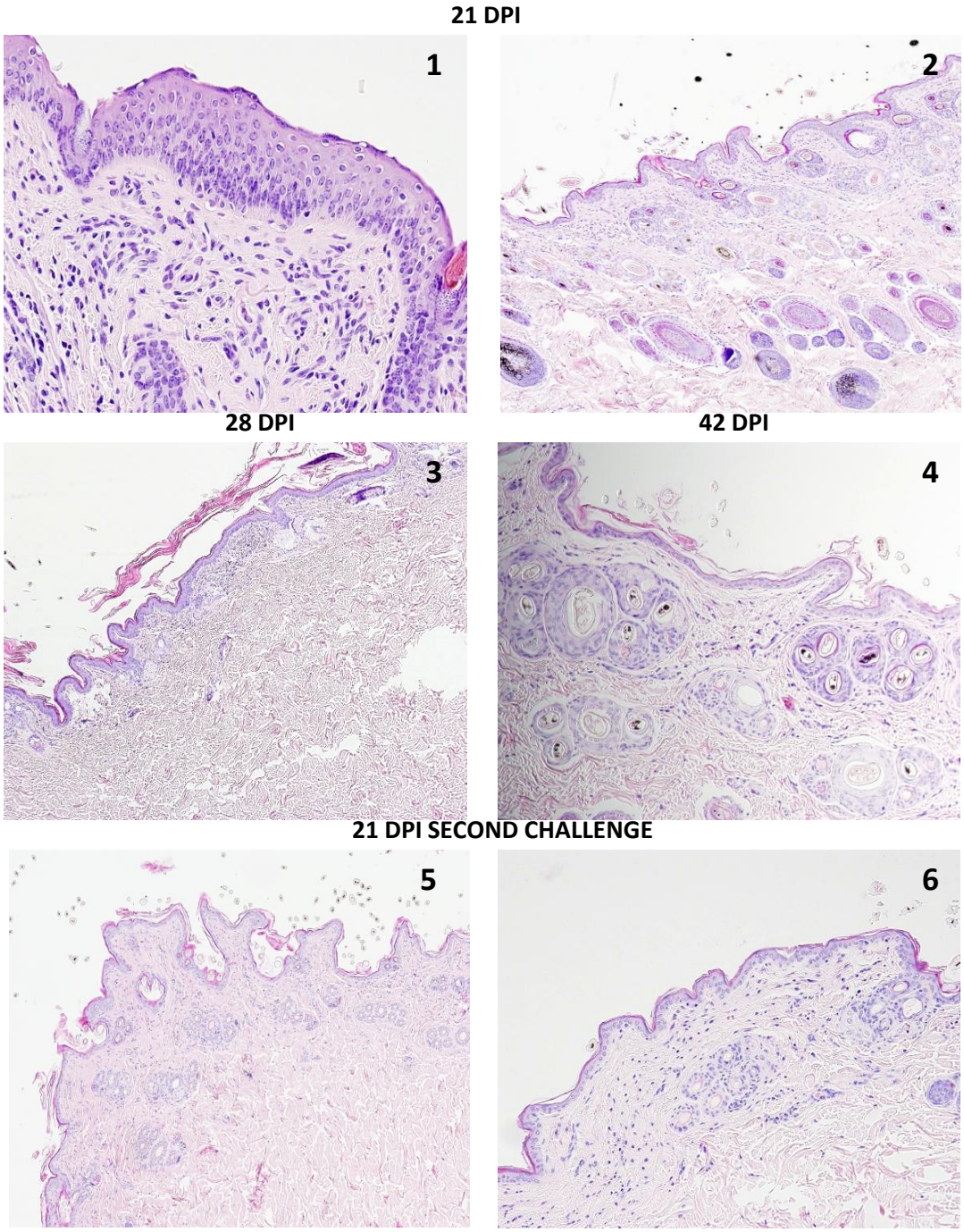


Figure 5.16 Section of primary lesion from SQPV scarified and mock scarified skin for long term infection. Haematoxylin and eosin staining.

1) Squirrel P1, SQPV scarified skin. Mild hyperplasia and some keratinocytes showing hydropic degeneration. Presence of lymphocytes in dermis. Original magnification x 200. **2)** squirrel G1: mock scarified skin. Normal appearance of skin with very mild inflammatory infiltrate. Original magnification x 40. **3)** Squirrel O1, SQPV scarified skin. Normal appearance of skin. Mild inflammatory infiltrate in the superficial dermis. Original magnification x 40. **4)** Squirrel J1, SQPV scarified skin. Normal appearance of skin. Original magnification x 100. **5)** Squirrel N1, SQPV scarified skin. Normal appearance of skin. Original magnification x 40. **6)** squirrel N1: mock scarified skin. Mild inflammatory infiltrate. Original magnification 1x 100.

5.9 Immunohistochemistry

In order to assess the distribution of viral antigens in the SQPV scarified skin at different time points post infection, immunohistochemistry (IHC) was performed (§**2.8**) on scarified, digital and eyelid skin samples. IHC focused mainly on the first 10 DPI for the scarified skin (**Figures 5.17-A-**,**-B-**,**-C-**,**-D-** and**-E-**) and for this reason results are only shown for the short term infection with some follow up analysis for eyelid and digital skin.

Following scarification, SQPV antigen was detected within epidermal cells by IHC from two to eight DPI in all the infected animals with differences between individuals in the degree of labelling. This may point to replication of the virus. The viral antigen labelling was generally associated with histopathological features such as hyperplasia and ballooning degeneration in the epidermis (e.g. **Figures 5.17–A-** slide **c**, and –**B-** slides **i** and **k**).

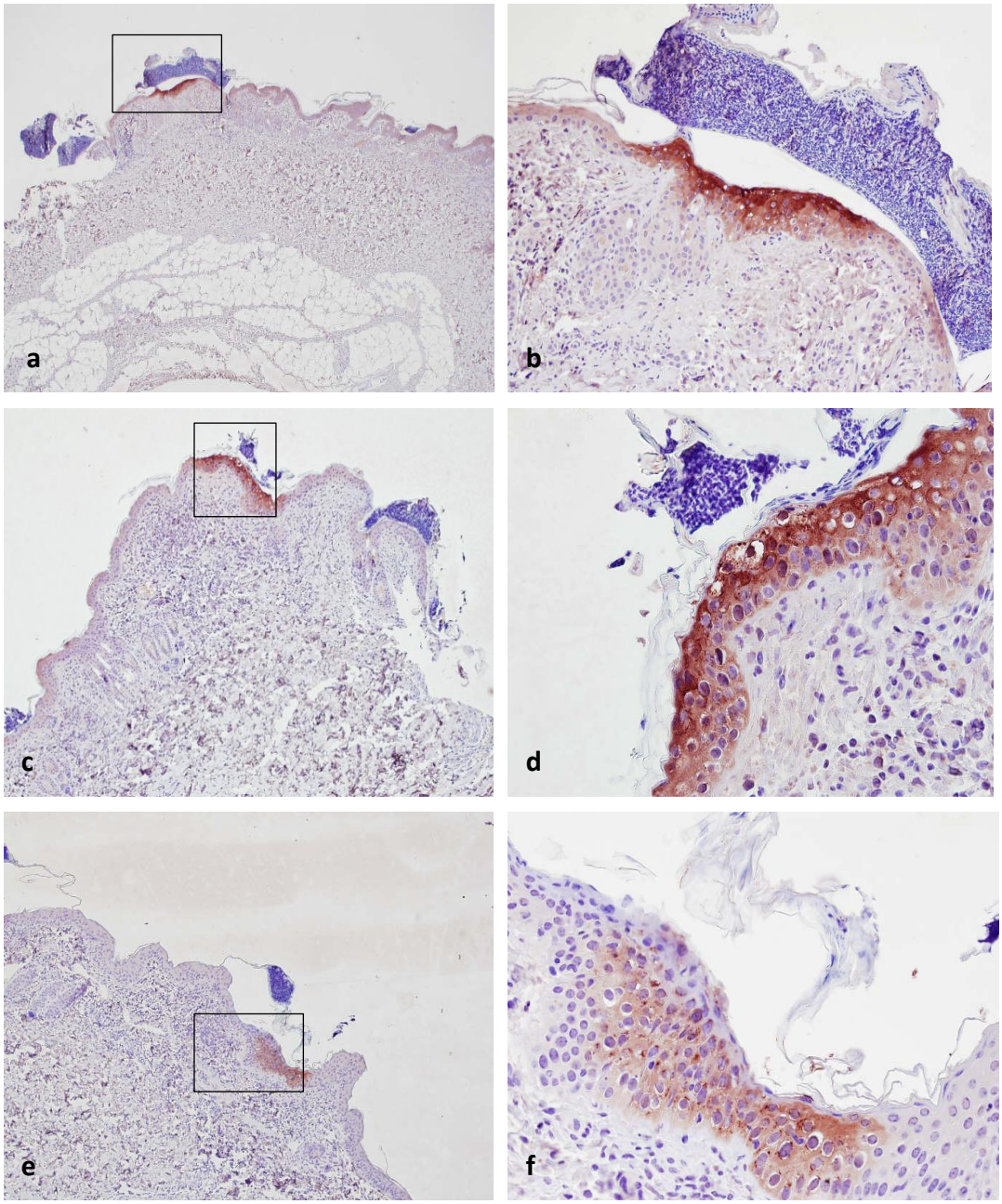


Figure 5.17-A- Immunohistochemical localization of SQPV antigens in SQPV scarified skin 2 DPI.

SQPV immunohistochemical labelling (brown stain). SQPV antigen is restricted to the epidermis with stronger positivity within the cytoplasm and membranes of the upper epidermal cells. The images on the right side represent a higher magnification of the section indicated by a black square on the left picture. Two top images: squirrel C2; two middle images: squirrel O2; two bottom images: squirrel V1.Original magnification: (a) x 20; (c, e) x 40; (b) x 100; (d, f) x 200

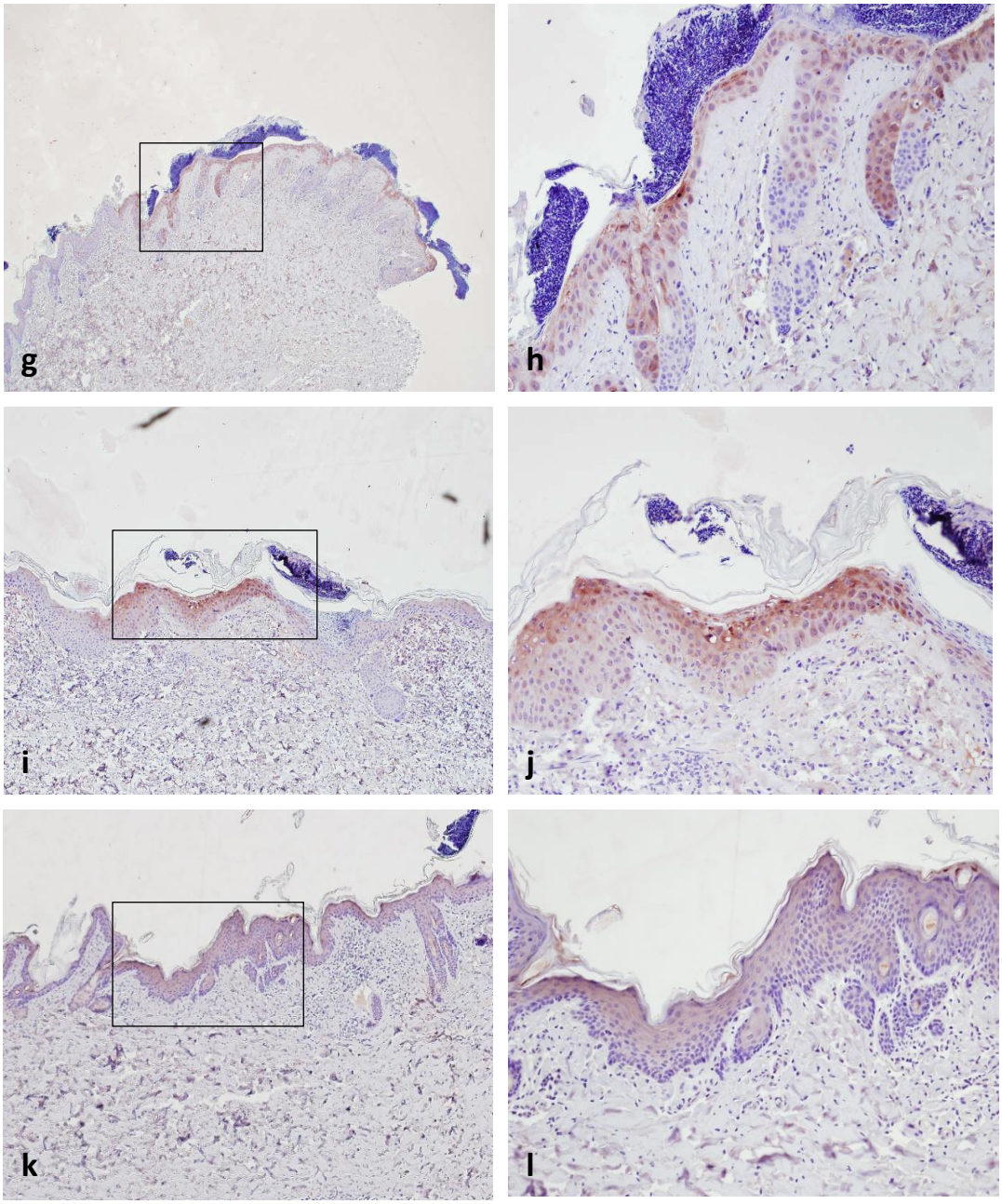


Figure 5.17-B- Immunohistochemical localization of SQPV antigens in SQPV scarified skin 4 DPI.

SQPV immunohistochemical labelling (brown stain). SQPV antigen is limited to the epidermis. Note the reduced and diffused staining in the upper spinous and granular layers compared to two DPI. The images on the right side represent a higher magnification of the images on the left. Two top images: squirrel B1; two middle images: squirrel H2; two bottom images: squirrel D1.Original magnification: (g) x 20; (i, k) x 40; (h, j, l) x 100

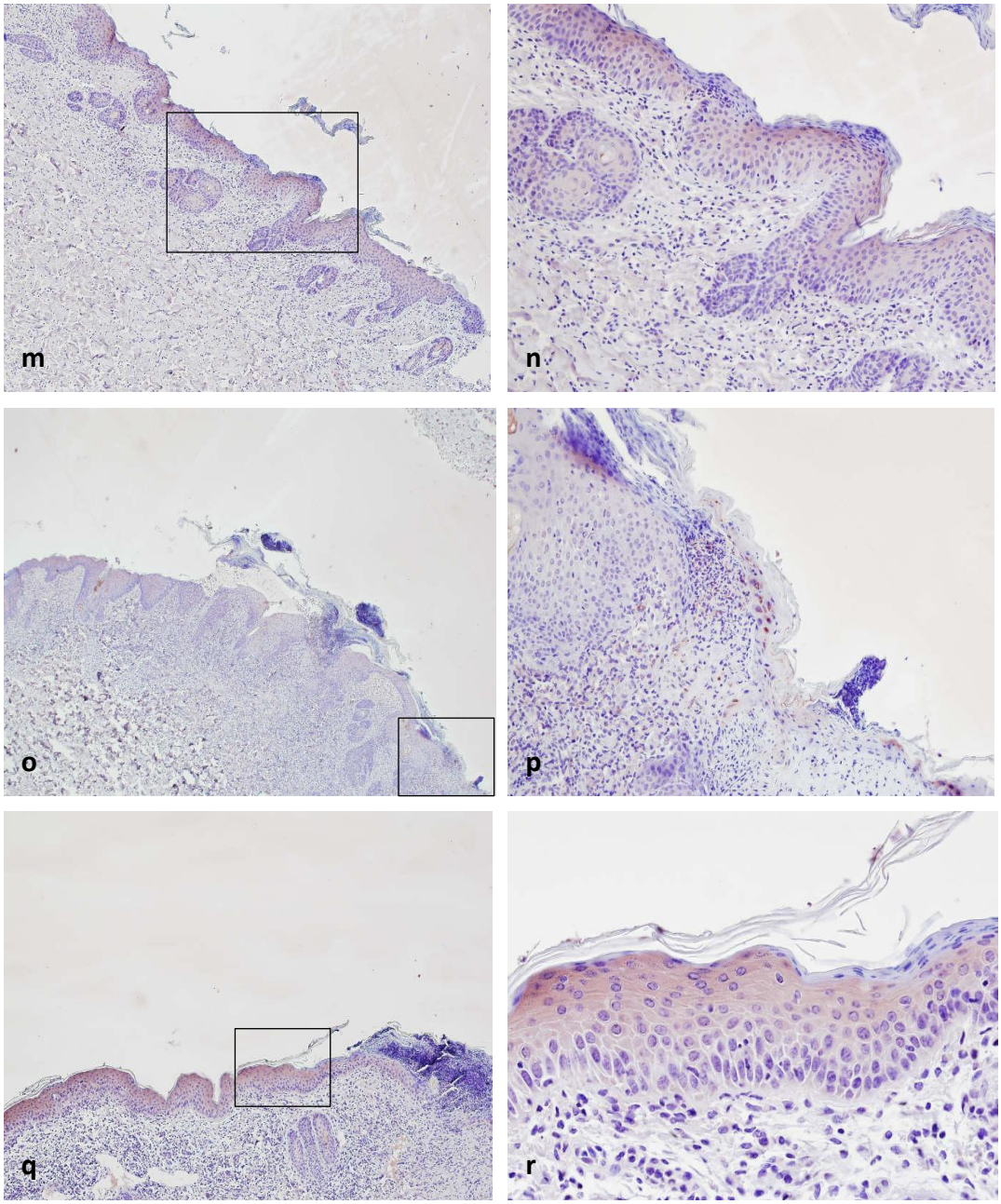


Figure 5.17-C- Immunohistochemical localization of SQPV antigens in SQPV scarified skin 6 DPI.

SQPV immunohistochemical labelling (brown stain). Minimal antigen is demonstrated within the epidermis cells with diminished detection toward the basal layer . The images on the right side represent a higher magnification of the images on the left . Location of right image is indicated by a black square. Two top images: squirrel C1; two middle images: squirrel A1; two bottom images: squirrel G2. Original magnification: (o) 20x; (m, q) 40x; (n, p) 100x; (r) 200x

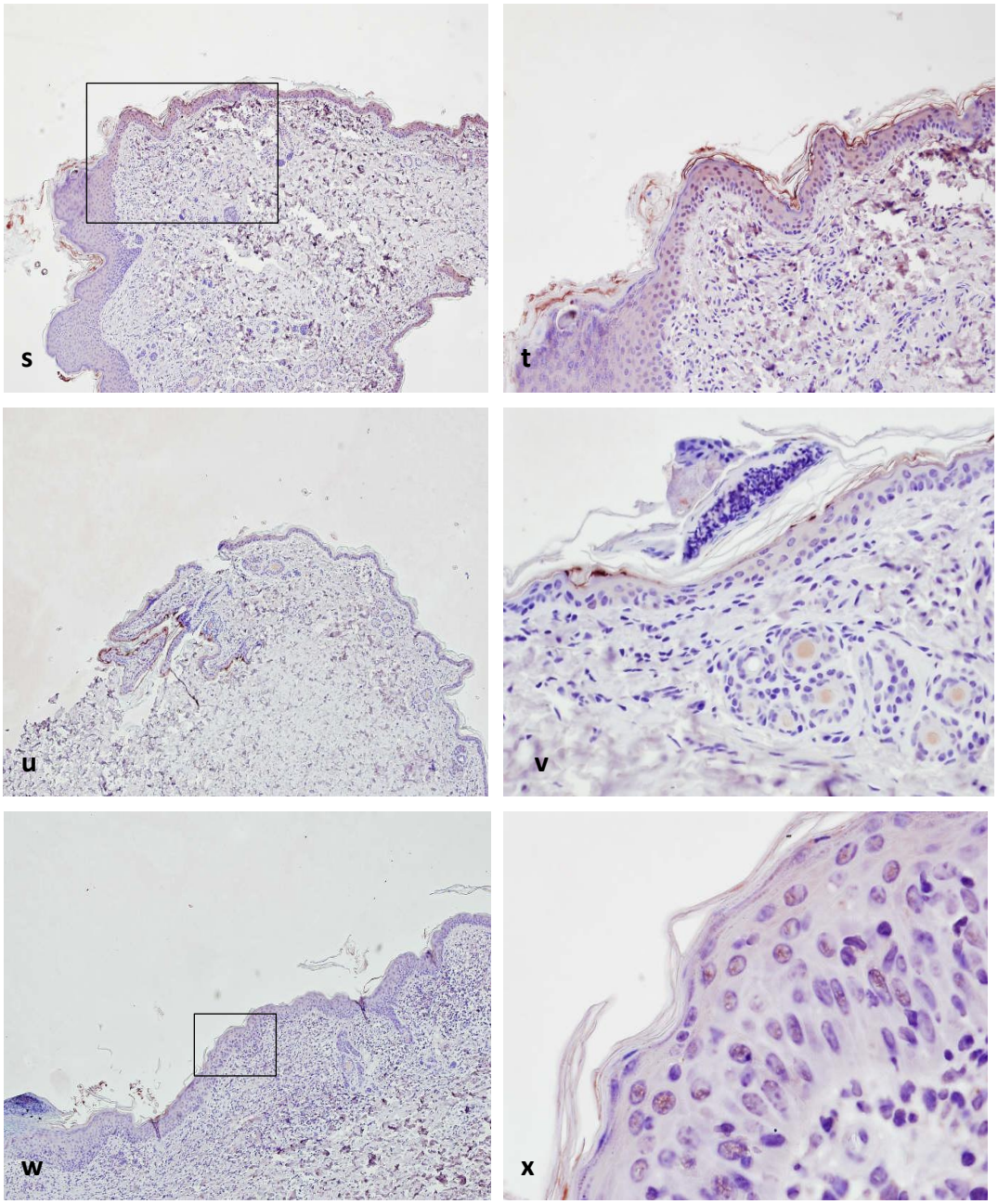


Figure 5.17-D- Immunohistochemical localization of SQPV antigens in SQPV scarified skin 8 DPI.

SQPV immunohistochemical labelling (brown stain). The brown stain shows areas of diminished viral antigen in the epidermis of skin sections. Note for images **t** and **v** viral staining is also present in the stratum corneum. Images **t** and **x** on the right side represent a higher magnification of the images on the left and their location is indicated by a black square. Images **u** and **v** show different skin sections from the same sample. Two top images: squirrel D2; two middle images: squirrel E2; two bottom images:squirrel J2. Original magnification: (s, v, w) 40 x; (t) 100 x; (v, x) 200 x

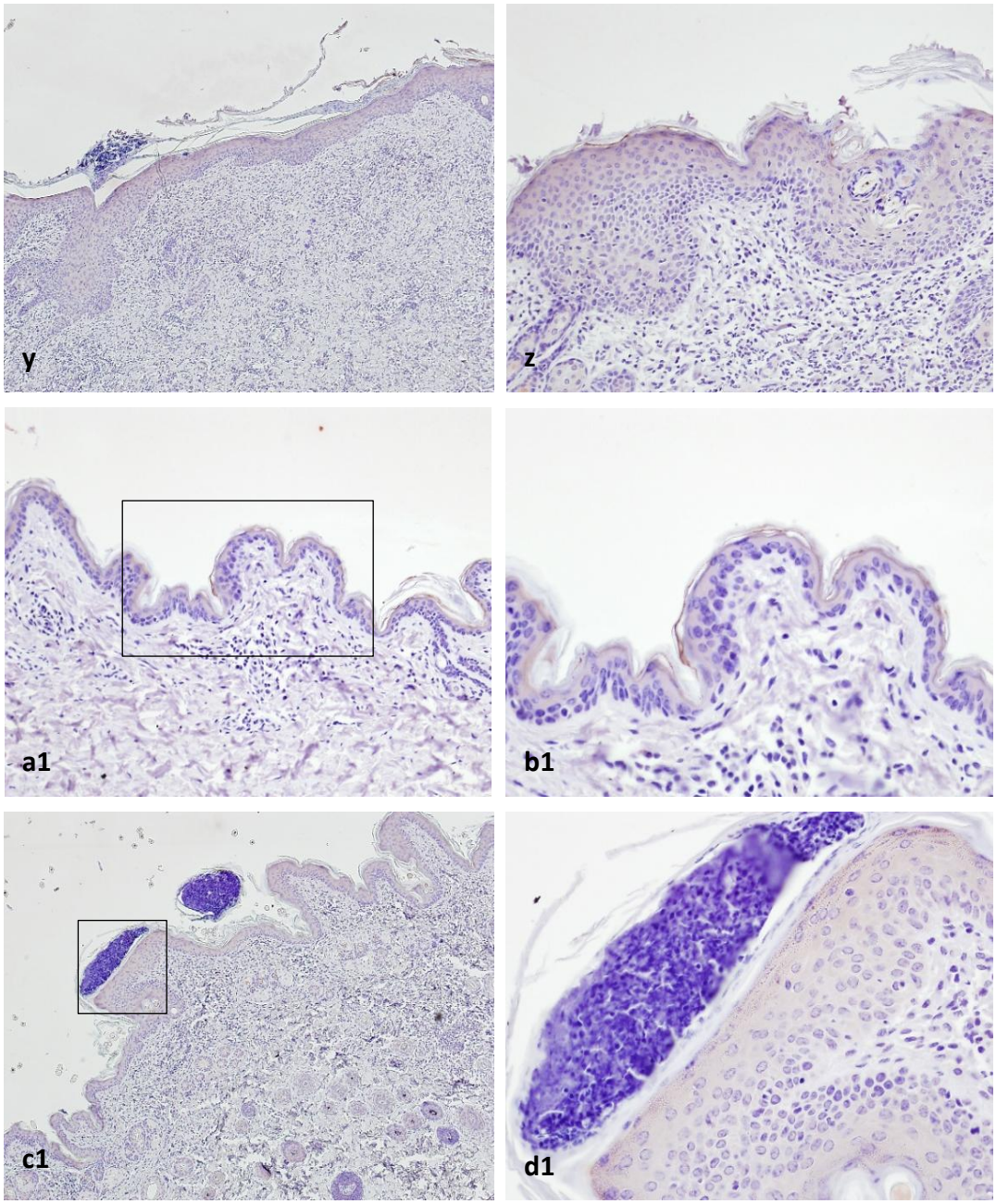


Figure 5.17-E- Immunohistochemical localization of SQPV antigens in SQPV scarified skin 10 DPI.

SQPV immunohistochemical labelling (brown stain). Skin samples show no further viral antigen detection by IHC at 10 DPI. Images b1and d1 on the right side represent a higher magnification of the images on the left and their location is indicated by a black square. Images y and z show different skin sections from the same sample. Top squirrel E1; centre squirrel F1; bottom squirrel I2. Original magnification (y, z, c1) 40 x; (a1) 100 x; (b1, d1) 200 x

Between days two and four post infection, SQPV antigen was distributed focally in the epidermis and labelling was more intense than in subsequent days. Virus specific labelling diminished thereafter but was still present up to eight DPI. At this point, viral antigen was observed diffusely in the cytoplasm of keratinocytes. At day 10 DPI virus labelling was very diffuse or absent in the scarified skin.

Viral antigen (with a lower degree of labelling compared to scarified skin) was also detected in two digital skin samples at day 21 after the second challenge and 42 DPI (squirrel Q1 and R1 respectively). No viral antigen was labelled in the eyelid skin of any of the samples tested (data not shown).

5.10 Discussion

Research describing the epidemiology and clinical features of SQPV infection has mainly focused on red squirrels (Keymer 1983; LaRose *et al*., 2010; Sainsbury & Gurnell 1995; Sainsbury & Ward 1996; Tompkins *et al*., 2002). There is little information on experimental infection of grey squirrels (Tompkins *et al*., 2002) and almost nothing is known about SQPV transmission. The experimental infection of grey squirrels in this study was therefore designed to mimic naturally occurring infection in order to investigate the clinical and pathological features of SQPV infection and the possible routes of virus transmission.

Concerns (from **Chapter 4**) about the viability of the virus present in the inocula to be used in the infection time course study were addressed before inoculation of the animals. Virus present in the inocula gave rise to CPE consistent with poxvirus infection when inoculated, and more importantly passaged, in foetal lamb skin cells (**Figure 5.2**). Furthermore electron microscopy of supernatant and cell debris from the passaged virus confirmed SQPV as the major, if not sole, infectious agent present in the cells (**Figure 5.4**).

Infected grey squirrels in this experiment, similar to findings by Sainsbury *et al*., (2000) and Tompkins *et al*., (2002) did not show any clearly visible clinical signs of infection or behavioural distress. The minor weight loss recorded during the long term infection was most likely linked to the stress caused by captivity rather than

being a consequence of infection. For the entire length of the experiment, the main signs of infection were subclinical and consisted of mild scab formation and mild erythematous dermatitis at the scarification site. However the primary lesions had healed within two weeks of infection without apparent progression to secondary sites.

These results were mirrored by histopathology and immunohistochemistry of tissues collected at different times post infection. Histopathology results from SQPV scarified skin showed a clear progression in pathological changes. In the first four days post infection, the majority of the changes affected the epidermis with most changes observed in the keratinocytes. Visible damage to these cells included ballooning degeneration and the onset of mild to moderate epidermal hyperplasia (acanthosis). The skin reaction to the virus was also associated with the presence of early dermal inflammatory infiltrate. The damage to the keratinocytes was directly associated with areas that showed the presence of a significant amount of viral antigen detected by IHC. As time progressed, the IHC indicated a reduction in viral antigen staining within the skin and an indication of the migration of the virus particles to the upper epidermis. The latter would lead to virus shedding from the skin surface.

Within the epidermis four days post infection there was increased evidence of hyperplasia as a consequence of the epidermal damage. The increase in inflammatory infiltrate in the dermis is likely to be a response to both the virus presence in the epidermis cells and the ongoing damage to the keratinocytes. The epidermotropic migration of lymphocytes (expected to be T-lymphocytes) into the epidermis was a further stage in the infection. The observed skin reaction could potentially result in an auto-immunologic “attack” by lymphocytes activated by virus (Shiohara & Mizukawa, 2005). Specifically, it may be that the complement of genes encoded by the virus that interact with the squirrel immune system may dampen the immune response in grey squirrels whilst being unable to do the same in red squirrels (Sainsbury, 2008). Consequently, histopathological changes in grey squirrels are not as well defined and lesions are more mild and localized compared to those of red squirrels.

In this study, for the first time, the serological response to SQPV infection was followed from pre-infection up to 42 days post infection. The majority of previous studies have been based on snap-shots with blood samples collected from culled grey squirrels (Bruemmer *et al*., 2010; Sainsbury *et al*., 2000). The current study therefore represents a controlled and continuous monitoring of antibody response following SQPV infection.

Strong antibody responses to poxviruses normally tend to be produced within a few days of infection (Smith & Kotwal 2002). However, consistent with Tompkins *et al*., (2002) only a minority of experimentally infected grey squirrels (8%) in this study had mounted an antibody response against SQPV within the first three weeks. Specifically, three animals did seroconvert after 12, 18 and 21 days respectively after the first challenge. For the few animals that did sero-convert the development of the antibody response therefore took longer than expected. However, after the second challenge the serological response was much more typical with two out of three animals sero-converting after one week and by the third week all three squirrels had shown a clear antibody response to SQPV infection. This suggests that the first challenge had successfully primed the animals to respond to subsequent virus challenges and re-confirmed that the virus present in the inoculum was likely to be viable.

The antibody ELISA results from the experimental infection also support findings by Talkington (2008) who showed that the current ELISA cut off of 0.2 OD_{450} has 99% specificity, indicating that almost all squirrels with a positive ELISA reading are indeed positive for SQPV. The corollary of this is that the current cut off therefore also has a high risk of producing false negative results. A revised OD_{450} reading of between 0.03 and 0.1 was therefore suggested as alternative cut off to differentiate between positive and negative samples (Talkington 2008).

A cut off of 0.03 OD_{450} would suggest that 16/37 (43%) grey squirrels in the current experiment (both short and long term combined) mounted an antibody response within three weeks following the SQPV scarification, with the first positive response just after six days post infection (**Table 5.5** squirrel G2). However the lower cut off of 0.03 would also lead to a larger number of false positive (lower specificity)

results. For example, squirrel L2 had an ELISA value of 0.047 OD_{450} despite the animal not being infected, and acting as a control. Taken together with its pre-infection value of 0.047 OD_{450} this either implies that the animal had already been exposed to SQPV in the wild, that it had been exposed during the experiment by aerosol or that the result represent a false positive reading. On the other hand if value of 0.1 was taken as the differentiating reading the status of squirrel B2 prior to the start of the experiment would need to be questioned. Squirrel (B2) tested positive (> 0.2 OD_{450}) 12 days post infection but already showed an ELISA OD_{450} of 0.121 at its arrival suggesting a potential prior exposure to SQPV.

In the south of Scotland, grey squirrels are being culled in order to prevent the spread of SQPV (Anon 2009). Efforts are focussed mainly on those areas where there is high sero-prevalence of SQPV infection in grey squirrels. It may be that this effort could be better informed by a revision of the ELISA cut off point which would allow more precise targeting of populations exposed to SQPV thus preventing further SQPV spread.

The development and use of other SQPV detection methods such as qPCR is also important. By the time it is possible to measure antibodies in grey squirrels, they may no longer be capable of transmitting the virus, nor can the method detect the presence of virus in the environment which is possible with qPCR assays. A further complication is that that red squirrels can develop disease and be a source of virus dissemination before they have developed antibodies against squirrelpox (see **Table 3.7** §**3.2.3.3**).

In agreement with histopathological findings, qPCR results in this study clearly indicated the presence of virus in specific target tissues in grey squirrels. The majority of positive results were obtained within the integumentary system (**Table 5.11**). No significant amount of virus was detected by qPCR in blood or the lymphoreticular system, suggesting there is no evidence of viraemia or systemic spread. This is also supported by the negative results on gross pathological examination and negative qPCR results for internal organs. However, a few popliteal lymph nodes were qPCR positive from the second day post infection up to 12 DPI (See **Table 5.12**). These positive results suggest that the presence of SQPV DNA in

popliteal lymph nodes from the limb on which the skin was scarified results from a local drainage from the scarification site rather than from haematogenous spread. The only positive contralateral popliteal lymph node came from Squirrel N2, (left popliteal lymph node). This lymph node drained the site of mock scarification, but in fact a lesion consistent with those obtained from the SQPV scarification sites did form at this location. The reason for this is not known, but it is believed that there was a high likelihood of cross-contamination from the SQPV-infected limb. The two positive values for the submandibular salivary gland could due to accidental ingestion of the virus (e.g. during grooming) which would also explain the only positive result for the stomach (squirrel N2).

Within the integumentary system, as expected, the largest amount of virus was present at the site of scarification. Gentle swabbing gave relatively high viral loads suggesting that the virus is most likely shed together with scab from the surface of the skin in accordance with the IHC results. Furthermore the qPCR results suggest that virus shedding from the skin surface will occur for longer than eight days (based on IHC results). Virus was detected on the skin surface until at least 18 days post infection. Positive results for ocular and oral swabs, and swabs from the nest boxes would be consistent with shedding of the virus in this manner. This would lead to contamination of the nest (and cage) environment. The presence of virus on the face, and particularly the area around the eyes, may however be through autoinoculation as a result of grooming behaviour.

There is some agreement between the results of the experimental infection reported here and the findings of Atkin *et al.*, (2010), such as the similarity between the virus distribution in red squirrels (see **Chapter 3**), and presence of SQPV in oral mucosa and lips of grey squirrels. However, the results of this study do not agree with the suggestion of viral spread to submandibular lymph nodes and to blood. Results from this experiment do not support the hypothesis of a systemic infection in grey squirrels. The suggested role of fleas (Atkin *et al.*, 2010) in the spread of the virus is also not supported for grey squirrels other than by possible mechanical transfer of the virus through external contamination of mouth parts, but even here the amounts of virus present in the skin of affected grey squirrels seem low for this to be a realistic possibility.

In general poxvirus infections follow two separate courses: a localized infection resulting in benign skin lesions, or a systemic infection, resulting in viral dissemination and often death (Smith & Kotwal, 2002). SQPV infection in grey squirrels appears to follow the former pattern and is subclinical, local and self-limiting.

CHAPTER 6

VARIATION IN THE GENOME ISOLATED FROM DIFFERENT OUTBREAKS OF DISEASE WITHIN THE UK

6.1 Introduction

Since its definitive characterization in 1981 more outbreaks of squirrelpox disease have been recorded in the UK. However, until now only one SQPV isolate from a single diseased red squirrel has been partially sequenced and deposited in the DNA sequence databases (GenBank).

This chapter outlines the results of a study comparing the nucleotide sequence of three different genes (located at the right and left-hand ends of the SQPV genome, as illustrated in **Figure 6.2**), carried out in order to assess the genetic diversity of SQPV isolates from different geographical locations within the UK collected between 1999 and 2008; and to investigate if the sequences of these genes varied from the virus originally isolated in 1984 and which has been passaged multiple times in cell culture.

6.2 SQPV outbreaks

Six SQPV isolates were selected from different geographical locations in the north of England and south of Scotland. Material comprised of scab or digital skin, were selected from confirmed SQPV clinical disease outbreaks in red squirrels. Four represented Scottish SQPV isolates (R597/07, R21/07, R05/08 and R811/08) and another two English outbreaks of disease (R1412/06 and R744/06) covering the time frame from 2006 to 2008. In addition, two further viruses were used in this study. These were 1296/99 and Sa1984, which are both from outbreaks of disease in England. Virus 1296/99 was isolated in 1999 in Gateshead in the north of England and is the reference isolate of SQPV, the sequence of which is deposited in the GenBank database under accession numbers DQ377804 and DQ377805 (McInnes *et al*., 2006). Virus Sa1984 is the cell culture-propagated virus corresponding to the

original SQPV first isolated by Sands and colleagues in 1984 (Sands J.J. *et al*., 1984; Scott *et al*., 1981a).The primers used for PCR in this study were based on the gene sequences of the SQPV 1296/99 isolate (§**2.5.3**). Details of the origin of each of the viruses are summarized in **Table 6.1** and **Figure 6.1**.

Table 6.1 Virus isolates used in this study

Virus isolates	Year of isolation	Location	Source	Serology results (SQPV ELISA)
1296/99	1999	Gateshead†	reference virus isolate (scab) *	No data available
R597/07	2007	NY156776	scab	+0.76
R21/07	2007	NY156776	scab	+1.38
R05/08	2008	NX836992	digital skin	Negative
R811/08	2008	NY360852	digital skin	Negative
R744/06	2006	NZ201869	scab	No data available
R1412/06	2006	NY795645	scab	Negative
Sa1984	1984	Norfolk†	cell cultured adapted virus **	No data available

* GenBank accession numbers: DQ3780377804 and DQ3780377805
** Culture adapted variant (Sands J.J. *et al*., 1984) of unknown number of in *vitro* passages of the SQPV isolate in a diseased red squirrel in 1981(Scott *et al*., 1981a)
†The exact location (OS Grid Reference) was not available for these isolates.

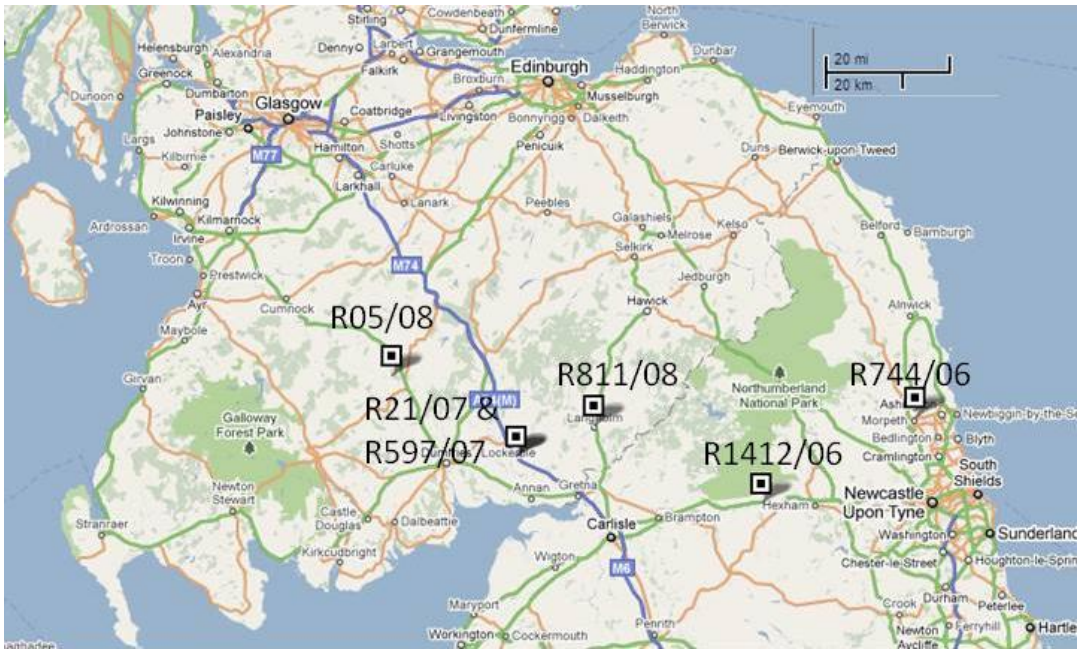


Figure 6.1 Map of part of the UK illustrating the location of six SQPV outbreaks from years 2006, 2007 and 2008 used for the study.

SQPV wild-type isolates are indicated with the R letter which corresponds to Red squirrel and identification number followed by the year after the oblique.

Real time PCR targeting gene I1L of the SQPV genome (see §**2.5.7**) was performed for each virus with the exclusion of virus Sa1984 and the reference virus 1296/99 (data not shown) in order to confirm the presence of SQPV.

6.3 Polymerase Chain Reaction (PCR) and cloning

PCR was used to amplify three different genes from the SQPV. Since sequence data was only available from one isolate of SQPV (1296/99) and therefore nothing was known about the potential sequence variation to expect, the genes used for this study were selected on the basis that in members of the *poxviridae* family the major sequence divergences tend to occur in the terminal, rather than the central region of the genome (Gubser *et al*., 2004). In addition since phylogenetic analysis had suggested that SQPV was closely related to the *Parapoxvirinae* the specific genes used in this study were selected based on knowledge of function and variability

within the *Parapoxvirinae*. Specifically, A6L corresponds to the gene for the major envelope protein. This gene is relatively well conserved within a species, but is sufficiently variable to differentiate between different species within the parapoxvirus genus. The exact functions of O8R and C1R ORFs are not known, but each is predicted to encode envelope glycoproteins which are amongst the five most variable proteins in *orf virus*, varying by up to 55% between isolates (Mercer *et al*., 2006). **Figure 6.2** is a schematic representation of the right and left hand ends of the SQPV genome and illustrates where the three genes are located.

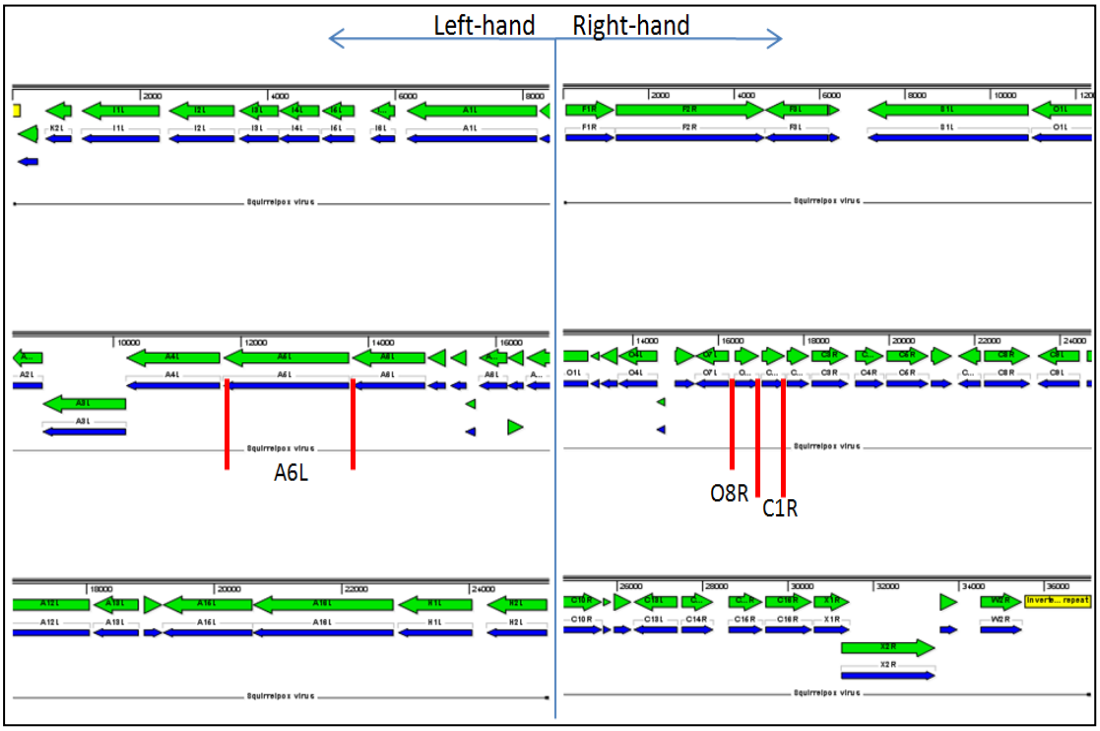


Figure 6.2 Schematic representation of the genetic map of the two ends of the SQPV genome.

On the left half and on the right half of the figure there are respectively the left-hand (LH) and the right-hand (RH) terminus of SQPV genome. The predicted open reading frames (ORFs) are indicated by the coloured arrows (green and blue). The relative location within the SQPV genome and length of the three genes of interested are encompassed within the red bars.

The resulting PCR products were first checked for the presence of an amplicon of the expected size for each gene (**Figure 6.3**) and then purified to remove excess primers, nucleotides, polymerase and salt as described in §**2.5.5**.

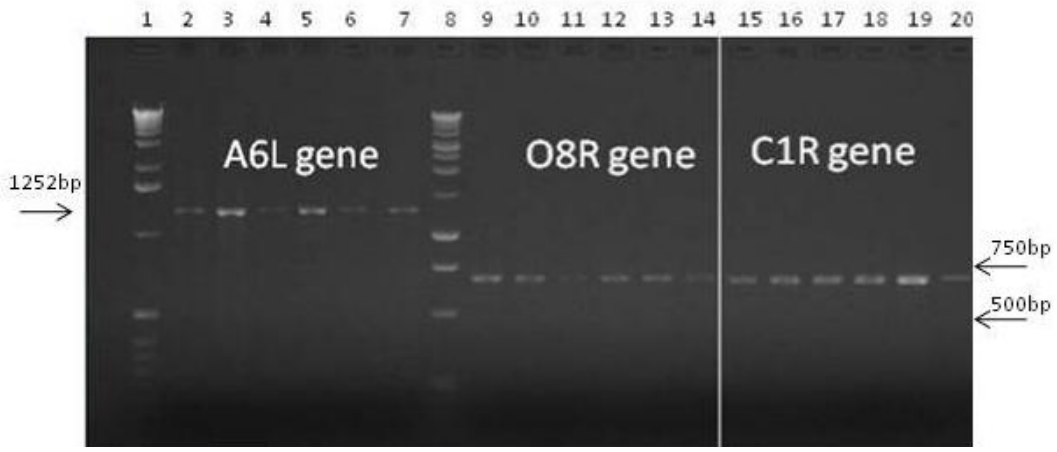


Figure 6.3 Agarose gel analysis of PCR products of the three different genes for the six clinical SQPV isolates.

1: 1kb DNA ladder (Invitrogen); 2 to 7: R597/99, R21/07, R05/08, R811/08, R744/06, R1412/06 amplicone for A6L gene; 8: 1kb DNA ladder (Promega)

When more than one band was visualized on the gel (data not shown), the band of the correct size was excised and DNA was purified from the gel. The PCR fragments were cloned into the pGEM®-T Easy Vector System and amplified in *E. coli* (see §**2.5.6,** §**2.5.8.1** and §**2.5.8.3**.).

6.3.1 Restriction endonuclease analysis of clones

The DNA from selected colonies was analysed on a 1.5% agarose gel after restriction enzyme digestion with *Eco* RI which cleaves the flanking sequences of the multiple cloning region releasing the insert. This step was performed in order to select which plasmid preparations contained the desired genes (**Figure 6.4**).

Figure 6.4 Example of clone restriction enzyme gel analysis of three genes of interest for two SQPV isolates.

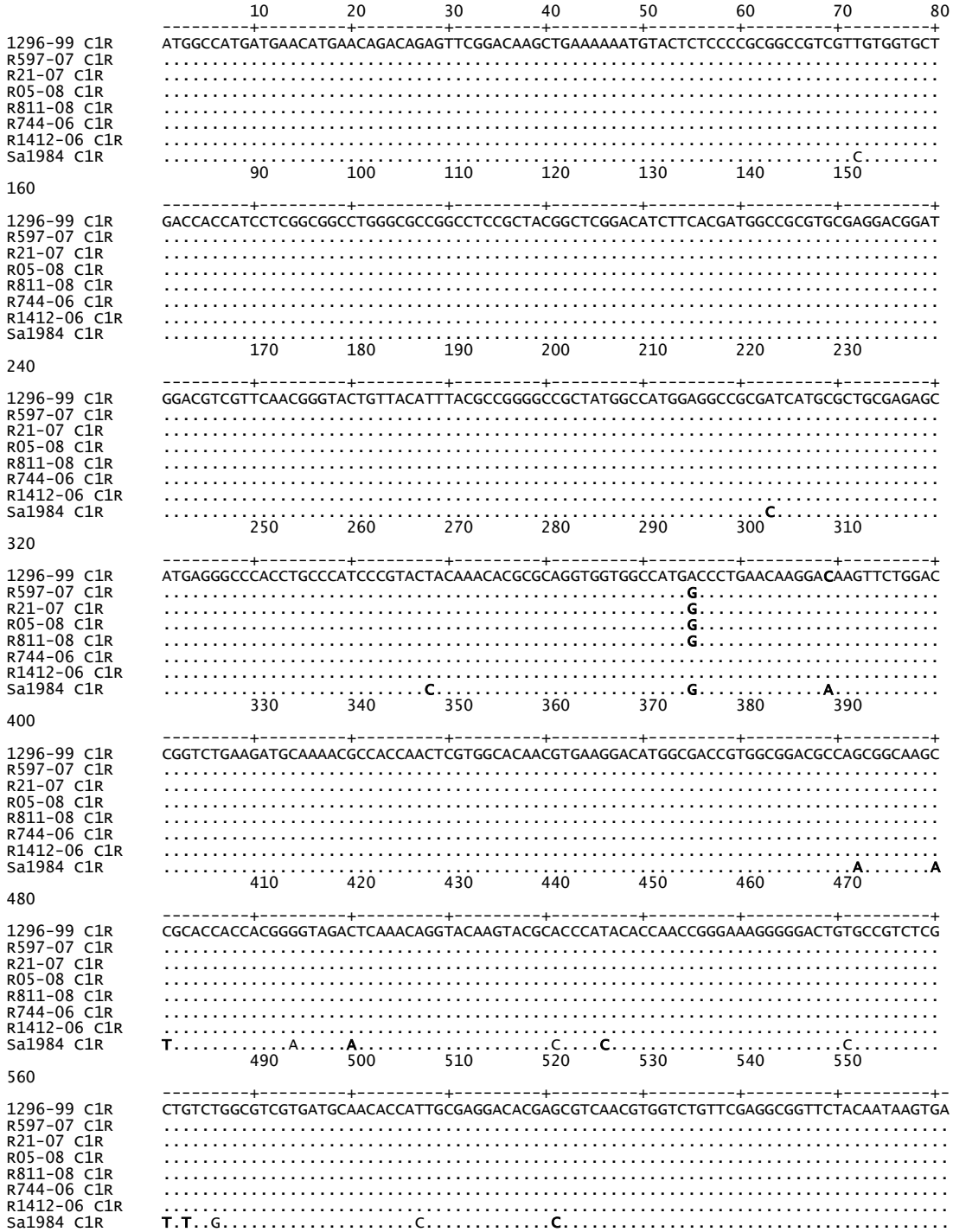
Eco RI was used to digest the recombinant plasmid DNA. 1and 2: two colonies from cloned gene O8R of R05/08; 3 and 4: two colonies of cloned gene A6L of R744/06 (the plasmid in lane 3 contained an insert of the wrong size); 5 and 7: two colonies from cloned gene O8R of R744/06 isolate; 6: 1kb DNA ladder (Invitrogen); 8 and 9: two colonies from cloned gene C1R of 744/06 isolate; one colony from cloned C1R gene of R05/08 isolate. pGEM®-T easy vector fragments are squared in orange. 1.5% agarose gel.

Digestion with *Eco* RI should result in two fragments being visible for each plasmid: ca. 3000bp for the linearised pGEM®-T Easy Vector, ca. 700bp for genes O8R and C1R and ca. 1300bp for gene A6L. Three clones of each gene from each virus were sent for sequencing by a commercial company (Eurofins MGW GmbH, Germany).

6.3.2 Nucleotides sequence analysis

Gene sequences from the seven SQPV isolates (six wild-type isolates and one from the cell-grown virus) were analysed using Seqman software (DNASTAR, lasergene Version 8.0) and a single consensus sequence produced for each gene from each virus. Comparison of these with the GenBank database, using the BLAST algorithm, confirmed each to have around 99% to 100% nucleotide identity to the reference sequence (1296/99) of SQPV. Multiple alignments of both the nucleotide and predicted amino acid sequences for each of the genes from the eight viruses were constructed using MegAlign software (DNASTAR, Lasergene Version 8.0) (**Figures 6.5, 6.6** and **6.7**).

A.


10 20 30 40 50 60 70 80
1296-99 C1R ATGGCCATGATGAACATGAACAGACAGAGTTCGGACAAGCTGAAAAAATGTACTCTCCCCGCGGCCGTCGTTGTGGTGCT
R597-07 C1R
R21-07 C1R
R05-08 C1R
R811-08 C1R
R744-06 C1R
R1412-06 C1R
Sa1984 C1R C
90 100 110 120 130 140 150
160
1296-99 C1R GACCACCATCCTCGGCGGCCTGGGCGCCGGCCTCCGCTACGGCTCGGACATCTTCACGATGGCCGCGTGCGAGGACGGAT
R597-07 C1R
R21-07 C1R
R05-08 C1R
R811-08 C1R
R744-06 C1R
R1412-06 C1R
Sa1984 C1R
170 180 190 200 210 220 230
240
1296-99 C1R GGACGTCGTTCAACGGGTACTGTTACATTTACGCCGGGGCCGCTATGGCCATGGAGGCCGCGATCATGCGCTGCGAGAGC
R597-07 C1R
R21-07 C1R
R05-08 C1R
R811-08 C1R
R744-06 C1R
R1412-06 C1R
Sa1984 C1R C
250 260 270 280 290 300 310
320
1296-99 C1R ATGAGGGCCCACCTGCCCATCCCGTACTACAAACACGCGCAGGTGGTGGCCATGACCCTGAACAAGGACAAGTTCTGGAC
R597-07 C1R G
R21-07 C1R G
R05-08 C1R G
R811-08 C1R G
R744-06 C1R
R1412-06 C1R
Sa1984 C1R C G A
330 340 350 360 370 380 390
400
1296-99 C1R CGGTCTGAAGATGCAAAACGCCACCAACTCGTGGCACAACGTGAAGGACATGGCGACCGTGGCGGACGCCAGCGGCAAGC
R597-07 C1R
R21-07 C1R
R05-08 C1R
R811-08 C1R
R744-06 C1R
R1412-06 C1R
Sa1984 C1R A A
410 420 430 440 450 460 470
480
1296-99 C1R CGCACCACCACGGGGTAGACTCAAACAGGTACAAGTACGCACCCATACACCAACCGGGAAAGGGGGACTGTGCCGTCTCG
R597-07 C1R
R21-07 C1R
R05-08 C1R
R811-08 C1R
R744-06 C1R
R1412-06 C1R
Sa1984 C1R T A A C C C
490 500 510 520 530 540 550
560
1296-99 C1R CTGTCTGGCGTCGTGATGCAACACCATTGCGAGGACACGAGCGTCAACGTGGTCTGTTCGAGGCGGTTCTACAATAAGTGA
R597-07 C1R
R21-07 C1R
R05-08 C1R
R811-08 C1R
R744-06 C1R
R1412-06 C1R
Sa1984 C1R T T G C C

B.

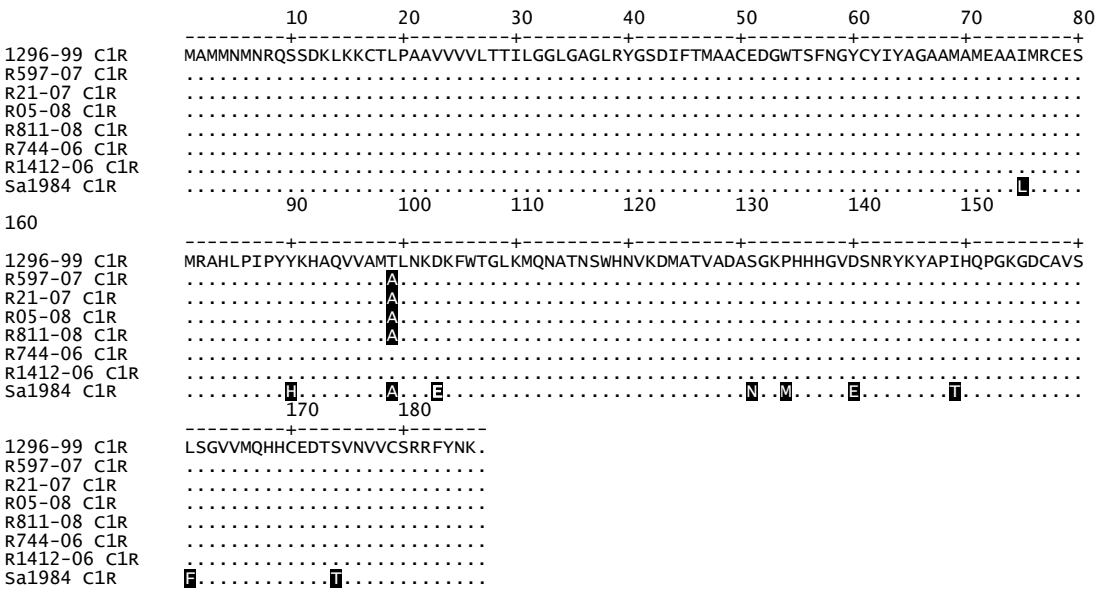
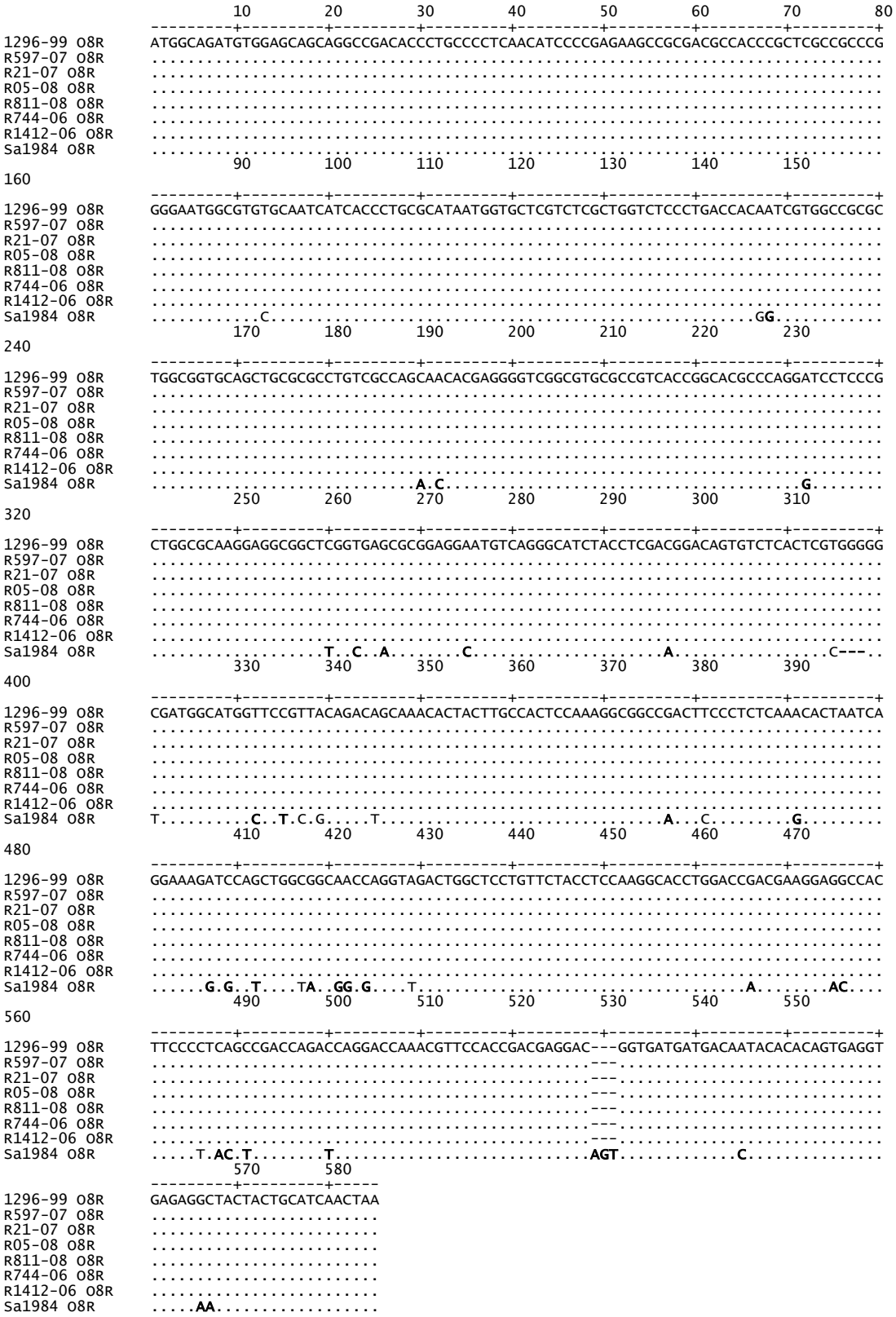


Figure 6.5 Multiple alignments of the nucleotide and predicted amino acid sequences of the C1R gene.

The SQPV C1R DNA (A) and predicted amino acid (B) sequences from the reference SQPV isolate (1296/99) are shown in the first line of each of the multiple alignments in comparison to which differences in the other virus isolates are noted. The single dots represent conserved sequence. (A) The nucleotides in bold represent bases that give rise to a corresponding change in the predicted amino acid sequence. Those base changes that are indicated, but not in bold, do not give rise to an amino acid change. (B) The predicted changes in amino acids from the reference sequence are highlighted.

A.


1296-99 O8R
R597-07 O8R
R21-07 O8R
R05-08 O8R
R811-08 O8R
R744-06 O8R
R1412-06 O8R
Sa1984 O8R

B.

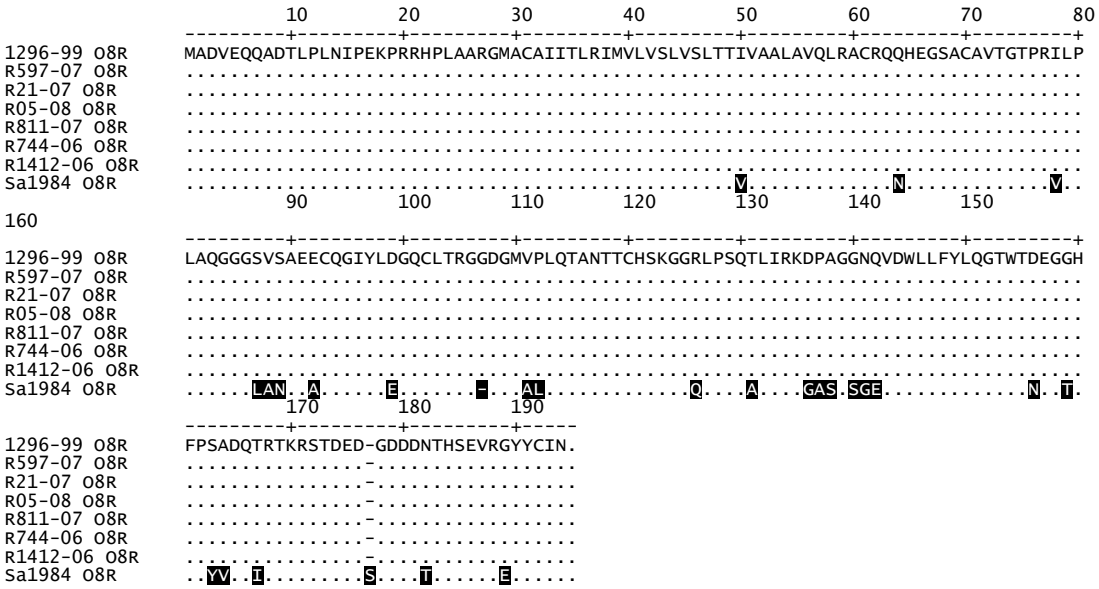
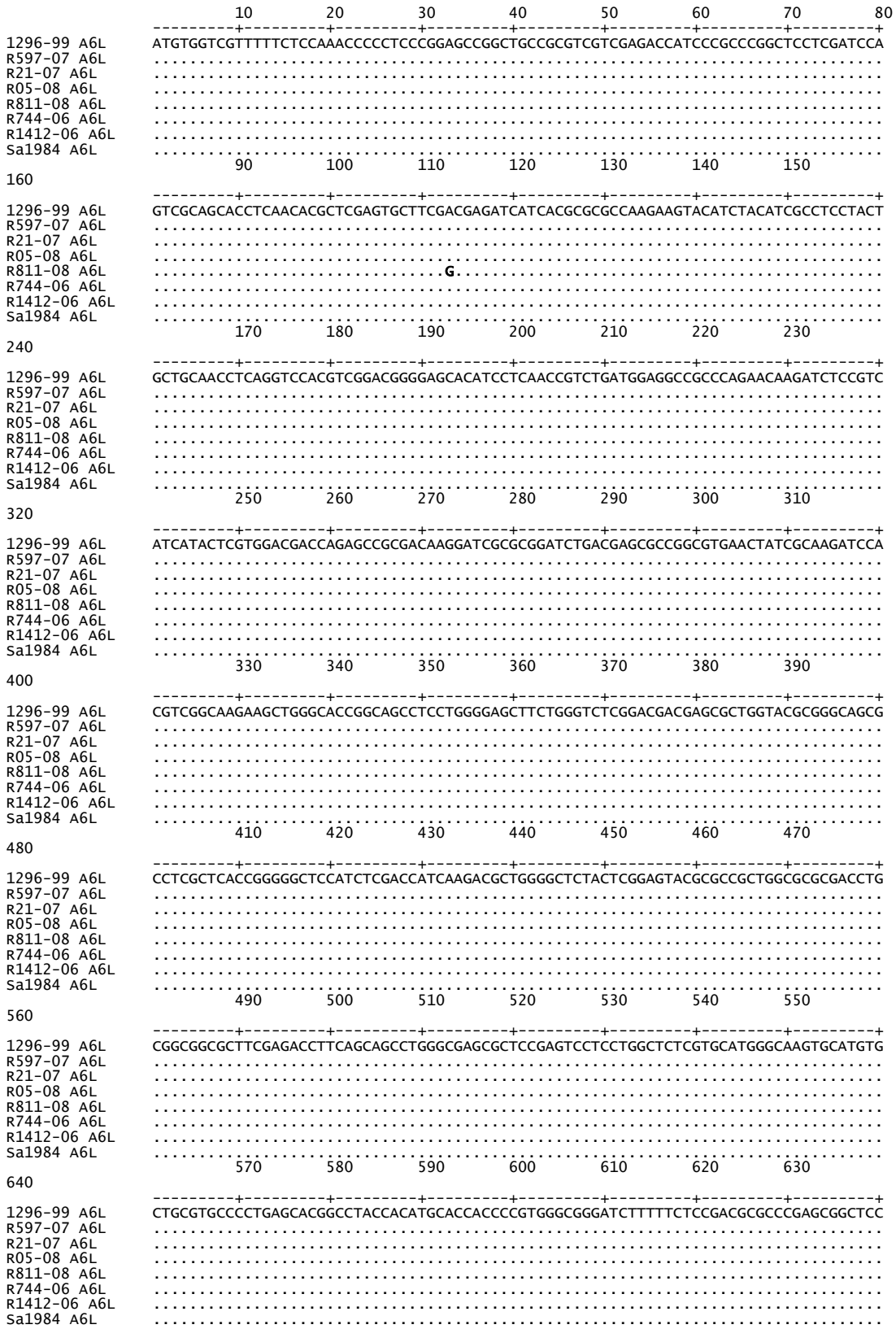


Figure 6.6 Multiple alignments of the nucleotide and predicted amino acid sequences of the O8R gene.

The SQPV O8R DNA (A) and predicted amino acid (B) sequences from the reference SQPV isolate (1296/99) are shown in the first line of each of the multiple alignments in comparison to which differences in the other virus isolates are noted. The single dots represent no changes in the sequence. Nucleotide or amino acid deletions (or insertions) are indicated by a dash. (A) The nucleotides in bold represent bases that give rise to a corresponding change in the predicted amino acid sequence. Those base changes that are indicated, but not in bold, do not give rise to an amino acid change. (B) The changes in predicted amino acids from the reference sequence are highlighted.

A.


10 20 30 40 50 60 70 80
1296-99 A6L ATGTGGTCGTTTTTCTCCAAACCCCCTCCCGGAGCCGGCTGCCGCGTCGTCGAGACCATCCCGCCCGGCTCCTCGATCCA
R597-07 A6L
R21-07 A6L
R05-08 A6L
R811-08 A6L
R744-06 A6L
R1412-06 A6L
Sa1984 A6L
90 100 110 120 130 140 150
160
1296-99 A6L GTCGCAGCACCTCAACACGCTCGAGTGCTTCGACGAGATCATCACGCGCGCCAAGAAGTACATCTACATCGCCTCCTACT
R597-07 A6L
R21-07 A6L
R05-08 A6L
R811-08 A6L G
R744-06 A6L
R1412-06 A6L
Sa1984 A6L
170 180 190 200 210 220 230
240
1296-99 A6L GCTGCAACCTCAGGTCCACGTCGGACGGGGAGCACATCCTCAACCGTCTGATGGAGGCCGCCCAGAACAAGATCTCCGTC
R597-07 A6L
R21-07 A6L
R05-08 A6L
R811-08 A6L
R744-06 A6L
R1412-06 A6L
Sa1984 A6L
250 260 270 280 290 300 310
320
1296-99 A6L ATCATACTCGTGGACGACCAGAGCCGCGACAAGGATCGCGCGGATCTGACGAGCGCCGGCGTGAACTATCGCAAGATCCA
R597-07 A6L
R21-07 A6L
R05-08 A6L
R811-08 A6L
R744-06 A6L
R1412-06 A6L
Sa1984 A6L
330 340 350 360 370 380 390
400
1296-99 A6L CGTCGGCAAGAAGCTGGGCACCGGCAGCCTCCTGGGGAGCTTCTGGGTCTCGGACGACGAGCGCTGGTACGCGGGCAGCG
R597-07 A6L
R21-07 A6L
R05-08 A6L
R811-08 A6L
R744-06 A6L
R1412-06 A6L
Sa1984 A6L
410 420 430 440 450 460 470
480
1296-99 A6L CCTCGCTCACCGGGGGCTCCATCTCGACCATCAAGACGCTGGGGCTCTACTCGGAGTACGCGCCGCTGGCGCGCGACCTG
R597-07 A6L
R21-07 A6L
R05-08 A6L
R811-08 A6L
R744-06 A6L
R1412-06 A6L
Sa1984 A6L
490 500 510 520 530 540 550
560
1296-99 A6L CGGCGGCGCTTCGAGACCTTCAGCAGCCTGGGCGAGCGCTCCGAGTCCTCCTGGCTCTCGTGCATGGGCAAGTGCATGTG
R597-07 A6L
R21-07 A6L
R05-08 A6L
R811-08 A6L
R744-06 A6L
R1412-06 A6L
Sa1984 A6L
570 580 590 600 610 620 630
640
1296-99 A6L CTGCGTGCCCCTGAGCACGGCCTACCACATGCACCACCCCGTGGGCGGGATCTTTTTCTCCGACGCGCCCGAGCGGCTCC
R597-07 A6L
R21-07 A6L
R05-08 A6L
R811-08 A6L
R744-06 A6L
R1412-06 A6L
Sa1984 A6L

```
                        650       660       670       680       690       700       710       720
                        ---------+---------+---------+---------+---------+---------+---------+---------+
1296-99 A6L             TGGGGTACTCGCGCACCCTCGACGCGGACTCGGTCCTGGGGTTCATCGAGTCCGCCACCACCAGCATCGACATGGAGCTG
R597-07 A6L             ................................................................................
R21-07 A6L              ................................................................................
R05-08 A6L              ................................................................................
R811-08 A6L             ................................................................................
R744-06 A6L             ................................................................................
R1412-06 A6L            ................................................................................
Sa1984 A6L              ................................................................................
                                730       740       750       760       770       780       790
800
                        ---------+---------+---------+---------+---------+---------+---------+---------+
1296-99 A6L             CTGTCCATCGTGCCGCTGGTGCGAAAAAACGACACGGTTAAATACTGGCCCGACATCTACAACGCCATCATCACCGCCGC
R597-07 A6L             ................................................................................
R21-07 A6L              ................................................................................
R05-08 A6L              ................................................................................
R811-08 A6L             ................................................................................
R744-06 A6L             ................................................................................
R1412-06 A6L            ................................................................................
Sa1984 A6L              ................................................................................
                                810       820       830       840       850       860       870
880
                        ---------+---------+---------+---------+---------+---------+---------+---------+
1296-99 A6L             CATCGACCGGAAGGTGCGCGTGCGCCTGCTGGTGGGGCTATGGCGCCATAACGACGTCTTCTCCATGGCCGCGGCGCGCA
R597-07 A6L             ................................................................................
R21-07 A6L              ................................................................................
R05-08 A6L              ................................................................................
R811-08 A6L             ................................................................................
R744-06 A6L             ................................................................................
R1412-06 A6L            ................................................................................
Sa1984 A6L              ................................................................................
                                890       900       910       920       930       940       950
960
                        ---------+---------+---------+---------+---------+---------+---------+---------+
1296-99 A6L             GCATCCAGGAGTTCGGCGTGGGCCACGCGGACGTCAGCGTGCGCGTCTTCTCCATGCCCGGGGGCGTGGGCGACGCCAAC
R597-07 A6L             ................................................................................
R21-07 A6L              ................................................................................
R05-08 A6L              ................................................................................
R811-08 A6L             ................................................................................
R744-06 A6L             ................................................................................
R1412-06 A6L            ................................................................................
Sa1984 A6L              ................................................................................
                                970       980       990       1000      1010      1020      1030
1040
                        ---------+---------+---------+---------+---------+---------+---------+---------+
1296-99 A6L             ATCAACAACACGAAGCTGATGGTGGTGGACGGCACCAAGGCGCACGTGACGGTGGCCAACTTCGACGGGACGCAGCACCT
R597-07 A6L             ................................................................................
R21-07 A6L              ................................................................................
R05-08 A6L              ............................................................A...................
R811-08 A6L             ................................................................................
R744-06 A6L             ................................................................................
R1412-06 A6L            ................................................................................
Sa1984 A6L              ................................................................................
                                1050      1060      1070      1080      1090      1100      1110
1120
                        ---------+---------+---------+---------+---------+---------+---------+---------+
1296-99 A6L             GCAGTACGCGTTCGTGAGCGTGAACAGCGTGGACCCGGGGGTCAGCAAGGAGCTGGCGGATGTCTTCGAGCGCGACTGGA
R597-07 A6L             ................................................................................
R21-07 A6L              ................................................................................
R05-08 A6L              ................................................................................
R811-08 A6L             ................................................................................
R744-06 A6L             ................................................................................
R1412-06 A6L            ................................................................................
Sa1984 A6L              ................................................................................
                                1130      1140      1150
                        ---------+---------+---------+--------
1296-99 A6L             ACTCGCCGTACAGCACGCCGCTACCGGCCTCAAAATGA
R597-07 A6L             ......................................
R21-07 A6L              ......................................
R05-08 A6L              ......................................
R811-08 A6L             ......................................
R744-06 A6L             ......................................
R1412-06 A6L            ......................................
Sa1984 A6L              ......................................
```

B.

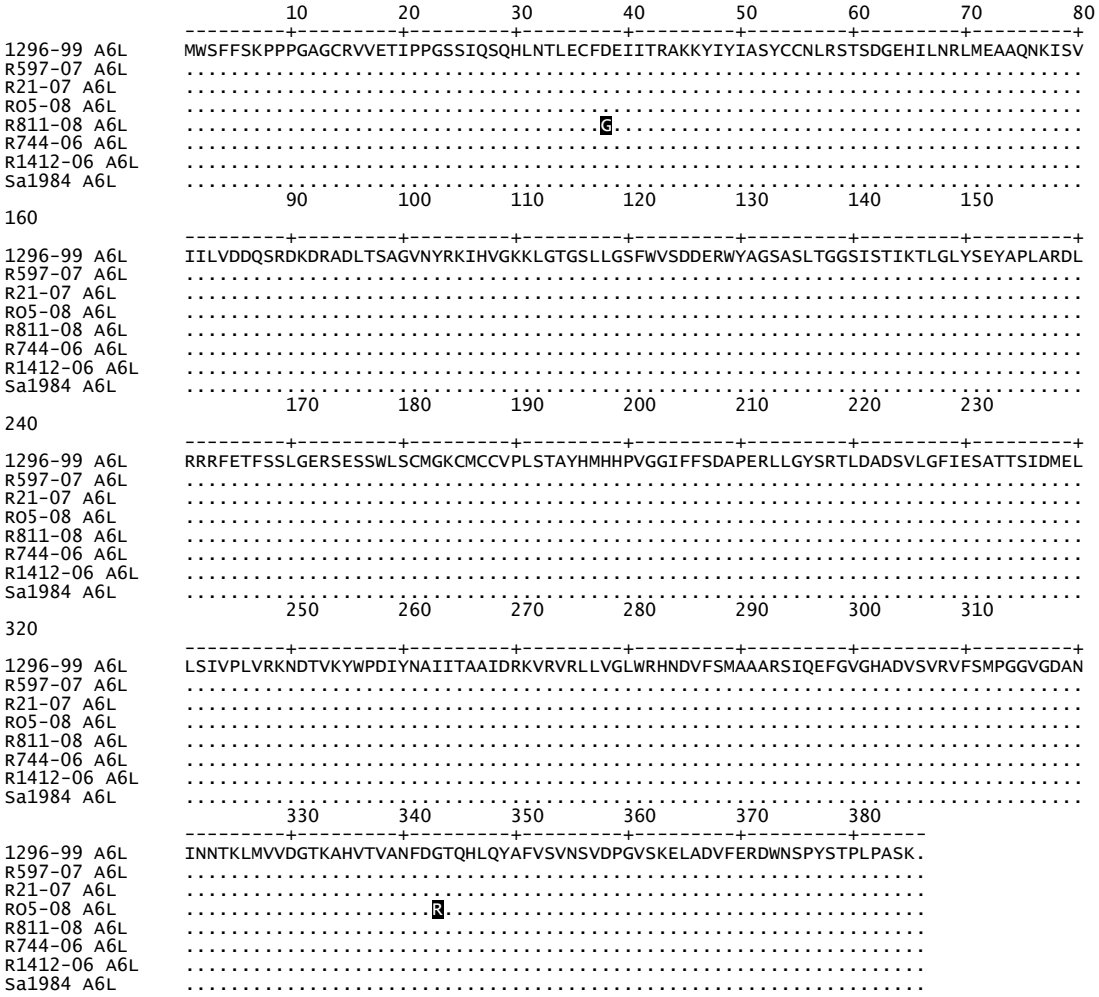


Figure 6.7 Multiple alignments of the nucleotide and predicted amino acid sequences of the A6L gene.

The SQPV A6L DNA (A) and predicted amino acid (B) sequences from the reference SQPV isolate (1296/99) are shown in the first line of each of the multiple alignments in comparison to which differences in the other virus isolates are noted. The single dots represent no changes in the sequence. (A) The nucleotides in bold represent bases that give rise to a corresponding change in the predicted amino acid sequence. (B) The changes in predicted amino acids from the reference sequence are highlighted.

The sequence analysis of the three genes was used to infer the relatedness of the viruses causing each outbreak of disease. Two of the isolates (R21/07 and R597/07) investigated were from the same outbreak, the first reported outbreak of disease in red squirrels in Scotland (McInnes *et al*., 2009). No differences were found between these two viruses. However there were very few differences in total between the seven geographically distinct SQPV isolates collected from outbreaks of disease between 1999 and 2008. Indeed of the approximately 2300 bases sequenced there were only 3 bases at which sequence variation was found; at one base within the C1R gene and at two bases within the A6L gene. All three base changes resulted in a change to the corresponding amino acid sequence. Interestingly, the base change in the C1R gene, which resulted in the substitution of alanine by threonine at position 99 in the predicted protein sequence, was common to all the isolates from Scotland in comparison to those from England, with the exception of the cell-adapted English strain Sa1984. In general, many more changes were observed in the cell-adapted strain Sa1984. For this virus the major amino acid changes occurred within the C1R and O8R genes. The Sa1984 C1R nucleotide sequence had 18 base changes in comparison to the reference sequence (1296/99) which resulted in 10 changes to the predicted amino acid sequence. For the O8R gene, 44 nucleotide bases changes were observed which resulted in 26 amino acid changes including one amino acid deletion (glycine) at position 107 and one insertion (Serine) at position 177. The variation in this gene sequence was particularly surprising given that the other six isolates did not show any change compared to reference isolate (1296/99).

Equally surprising was that for the A6L sequence no differences were found between the Sa1984 strain and the majority of the other viruses including the reference isolate. Indeed only two point mutations (**Figure 6.7**), one in each of two of the Scottish viruses, were found, but both did result in a change to the predicted amino acid sequence.

6.4 Discussion

The concern before starting this study was that the genes selected for comparison of isolates would not be sufficiently variable to allow meaningful interpretation. Initial results suggested that this indeed might have been the case since only three base changes were found in the 2301 bases of sequence determined for each of the seven wild-type isolates of virus. However, sequence from the Sa1984 cell adapted virus was more reassuring in that multiple base changes were found in the O8R and C1R genes (cf. the reference isolate) whilst no changes were found in the A6L gene. This corresponded to previous predictions and validated their choice as suitable candidate genes with which to carry out this study. It had been expected that the A6L gene would be relatively less variable between viruses, but that the O8R and C1R genes would be more variable. The fact that the only variation was seen between the cell-adapted strain and all the other viruses may mean that some questions remain about their suitability as target genes for this analysis, suggesting that different genes from the terminal region of SQPV genome should also be examined for potential changes. Nevertheless, in this limited study, some general conclusions can still be drawn from the data.

The results confirm that a single species of virus is responsible for the disease that is recognised in the UK as squirrelpox and that the SQPV circulating within the squirrel population in the north of England and the south of Scotland does not vary significantly between different geographical locations. This could, indicate a single source of virus or be an indication of the stability of poxvirus genomes. Whether this suggests that the SQPV UK genotype came from a single introduction of virus from America or that the same virus genotype was already present in the grey squirrels in America as a unique strain and was imported to the UK at different times and in different locations through several grey squirrel introductions (see **Chapter 1**) is unknown. However, the fact that the cell-adapted virus Sa1984 possessed multiple differences in the O8R and C1R genes might suggest that there is more than one genotypically distinct form of the virus circulating in the UK. Alternatively, it could be argued that isolate Sa1984 is representative of the SQPV circulating at the time of its isolation (1981) and that in the subsequent 18 years it accumulated multiple mutations to become the form of virus isolated in 1999 (1296/99). A similar mutation

rate, however, was not observed between the 1296/99 isolate and those isolated from 2006-08, a period spanning approximately 10 years, arguing against this scenario. There is also the possibility that the unique sequence of the Sa1984 strain is a consequence of virus adaptation and passage *in vitro,* but this could not be tested since the early passages of the virus have been lost.

In conclusion, relatively low variation in the nucleotide sequence across the SQPV isolates is not consistent with the hypothesis of co-circulation of multiple SQPV genotypes, resulting in diverse geographically defined virus strains, but rather the presence of a restricted pool of SQPVs in the UK. Further investigation of more clinical SQPV isolates from different parts of the British Isles might provide evidence to the contrary.

CHAPTER 7

GENERAL DISCUSSION

7.1 Discussion

Over the last 100 years, Great Britain has seen a dramatic decline in the native red squirrel, which has now disappeared from the vast majority of the country with just a few isolated populations remaining. In contrast, the imported grey squirrel has greatly expanded its range, with adverse consequences for the red squirrel which is heading towards extinction from the UK mainland (Parrott *et al*., 2009). However, although several reports suggested disease as a major threat to the red squirrel (Keymer 1974; Vizoso, 1970), it was only relatively recently that a viral agent responsible for red squirrel pox disease outbreaks was identified (Scott *et al*., 1981a). It took another 20 years before research established the importance of this epizootic disease in the wider context of disease-mediated competition with grey squirrels, with the result that conservation strategies for the red squirrels changed to take account of this. Red squirrels have been protected under the UK Wildlife and Countryside Act since 1981, but only since 1997 have they been included in the UK Biodiversity Action plan (BAP) Priority Species List. This includes an individual Special Action Plan to promote policies to reduce the current rate of biodiversity loss (Macdonald *et al*., 2011). Despite this recognition SQPV still represents one of the biggest threats to native red squirrel conservation in UK.

After their introduction to the country in 1876 (Middleton, 1930), the grey squirrel population expanded rapidly to the detriment of the red squirrel population and it is the transmission of SQPV, carried by grey squirrels, into the immunologically naive red squirrels, that appears to be the major factor in this take over. It has been shown that where sympatric populations of red and SQPV seropositive grey squirrels are found, the decline of red squirrels can be 17- 25 times faster than in areas (e.g. Italy) where SQPV has never been detected (Rushton *et al*., 2006).

Prior to 2005, SQPV seropositive grey squirrels were recorded only in England and Wales. However, as the geographical range of the seropositive grey squirrels expanded northwards, surveillance revealed the emergence of the threat from SQPV in southern Scotland for the first time. Within 2 years, the disease had become evident in the red squirrel population putting the last remaining stronghold of the red squirrel in the UK in jeopardy (McInnes *et al*., 2009).

Despite it being 30 years since SQPV was first identified as causing disease in red squirrels, remarkably little has been discovered about the epidemiology of the virus in red and grey squirrels or the transmission within and between the species. The work presented here was carried out to investigate SQPV infection in both squirrel species and to assess possible virus transmission routes and dynamics in free-ranging squirrel populations by using an experimental infection model in grey squirrels. This was seen as an essential part of the longer term challenge of developing a SQPV vaccine. The examination of natural cases of disease in red squirrels was the first comprehensive qualitative and quantitative analysis of virus dissemination in squirrel tissues and the experimental SQPV infection in grey squirrels allowed a similar analysis of dissemination and shedding of SQPV in its natural host. For the first time a variety of diagnostic methods (e.g. ELISA, qPCR and immunohistochemistry) were employed concurrently to follow the infection in squirrels.

The qPCR results presented here confirmed that the amount of viral DNA detected in infected red squirrels was always greater in skin samples compared with internal organs, suggesting that it is the most likely site for virus replication. This was also reinforced by evidence from post mortem examinations with skin being the only tissue exhibiting obvious pathological changes, characterized by an erythematous exudative dermatitis, often associated with haemorrhagic ulceration. The absence of gross and histopathological abnormalities in the internal organs was taken as evidence of the lack of systemic disease, although not necessarily lack of systemic dissemination of virus. In naturally infected animals rapid disease progression, with multiple skin lesions, leads to high mortality rate, but the cause of death in red squirrels affected by SQPV remains a mystery.

It has been hypothesised (Carroll *et al*., 2009) that the epidemiological characteristics of squirrelpox resembles those of other virulent poxviruses, and in particular mousepox (*Ectromelia virus*, ECTV) in mice. As a result it has been suggested that SQPV was likely to have an incubation time of no longer than 15 days with the course of disease on average lasting approximately 10 days. Based on this it was expected that naturally-infected red squirrels would exhibit an initial viraemic phase with the involvement of one or more organs followed by a secondary viraemia when the virus is released from organ(s) resulting in lesions at distal sites in the skin (Esteban & Buller, 2005). However, not only did the post mortem findings suggest that, in contrast to mousepox infection, no abnormalities are found in the liver or spleen of affected red squirrels, but qPCR results also failed to demonstrate substantial quantities of viral DNA in these organs (as discussed in **Chapter 3**), suggesting that the pathogenesis of SQPV in red squirrels is substantially different to that of ECTV in mice.

The spleen is a well perfused organ and it may be that the amount of viral DNA detected in the spleen of squirrels, and indeed in other internal organs in which viral DNA was detected, is a reflection of the amount of virus present in the blood of infected animals (Bowden *et al*., 2008).

In addition, a recent paper (Atkin *et al*., 2010) suggested that in red squirrels, squirrelpox is characterized by systemic spread of the virus. In their study, nested PCR and real-time PCR (SYBR® Green technology) were used for detection and quantification of viral DNA in tissue samples from diseased and apparently healthy red and grey squirrels. As in the present study, they found viral DNA in a number of organs, but with skin (e.g. lip and antebrachial skin) containing the largest amounts. Importantly, however, they reported that viral DNA was also detected in the blood and lymph nodes of grey squirrels and suggested a viraemic phase in the pathogenesis of SQPV infection and a possible role for fleas in transmission of the virus. Other studies, however, have questioned the role of fleas in transmission of SQPV mainly due to the seasonality of disease and have suggested that direct contact between infected squirrels is the more likely route of infection (Bruemmer *et al*., 2010; Carroll *et al*., 2009; Sainsbury, 2008).

Biting insects had been considered as a possible SQPV vector because it was known that within the *Leporipoxvirus* genus, *Squirrel fibroma virus* (SQFV) and *Myxoma virus* (MYXV) have been shown to be transmitted by insect vectors (Buller & Palumbo, 1991; Robinson & Kerr, 2001; Terrel *et al*., 2002). The early stages of myxomatosis and fibromatosis are both characterized by blepharitis and proliferative lesions on the eyelids. The reason for this has been suggested to lie in a possible preference for hairless skin by the arthropod vectors resulting in the initial inoculation site being the eyelids (Terrel *et al*., 2002). To counter this, conjunctivitis and periocular oedema are identified as discriminating clinical or post mortem findings in the early stage of monkeypox virus infection in prairie dogs and other rodents, where insects are not believed to be involved in the epidemiology of infection. Instead, transmission in this case is thought to be by aerosol (Langohr *et al*., 2004).

SQPV infection in red squirrels is characterized by mild facial oedema and the eyelids are often covered with haemorrhagic crusts and scabs (see **Chapter 3**). Although the qPCR results here suggest that systemic spread of the virus within the squirrel may be a factor in the appearance of secondary lesions distal to the site of infection, it may be that fleas and other biting insects could be involved, but not in the conventional sense. Infestation with ectoparasites is a common occurrence in wildlife. It is possible that the distress caused by insect bites exacerbates grooming behaviour in the animals resulting in micro abrasions in the skin, a likely predisposing factor in poxvirus infection. Mechanical transfer of the virus to these micro abrasions, either via the feet of the squirrel itself or indeed maybe from the mouthparts of the biting insects could result in the formation of lesions.

A recent outbreak of SQPV disease in red squirrels in Ireland used the information gathered herein to help in the diagnosis of infection. Two of the animals submitted for testing had been euthanased because they had exhibited signs of lethargy and incoordination. Neither had any signs of dermatitis and both appeared outwardly healthy. Post mortem examination of these animals did not reveal any gross pathological changes internally other then periocular oedema associated with alopecia, although histopathology did show a mild ballooning degeneration of the eyelid skin. PCR results from the eyelid skin however, were positive for SQPV

(Dagleish and McInnes, personal communication, 2011). It is thought that these squirrels were in the very early stages of disease and that lethargy and periocular oedema could be used for an early diagnosis of SQPV infection. More importantly, taking all the results together, an initial virus tropism for the eyelid skin in naturally infected red squirrels was indicated. For that reason confirmation of infection in red squirrels is now routinely carried out by performing the PCR assay on submitted eyelid skin samples.

The drawback to investigating squirrelpox in naturally infected red and grey squirrels is that it is not known when the animals were infected in relation to when they died or were euthanased. It was for this reason that an experimental infection of grey squirrels was performed. Tompkins *et al*., (2002) and Thomas *et al*., (2003) had previously shown that experimental infection of grey and red squirrels leads to sero-conversion of both species, but that only red squirrels succumbed to disease. However, the grey squirrels were not euthanased until 6 weeks after a second challenge infection, by which time, if the initial infection had resulted in some degree of immunity in these squirrels, the presence of the virus and any disease it caused may have been lost.

The results here confirm that scarification and topical application of the virus can be used to infect grey squirrels with SQPV, that infection is largely characterized by subclinical disease, and that the skin is the most likely tissue for virus replication. This should be viewed in the light that experimental models are rarely able to mimic exactly what happens in natural infections. Nevertheless, immunolabelling of scarified skin sections from the experimentally infected grey squirrels (**Chapter 5**) demonstrated the presence of viral antigen within the cells of the epidermis demonstrating that the grey squirrel is both susceptible and permissive for SQPV. These results strongly suggest that the virus is capable of replicating in the epidermal cells (probably keratinocytes) since antigen was detectable for up to eight days after challenge. However, typical poxviral inclusion bodies were not numerous and not as clearly defined as those in infected red squirrel tissues; further, the histopathological changes were less severe compared to those in infected red squirrel skin samples. A low level of viral DNA was detected by qPCR in skin samples distant from the site of scarification (e.g. digital, eyelid) and oral and ocular surfaces and, in some

animals, in the popliteal lymph nodes (which drain the scarification area). However, as suggested previously, autoinoculation of the virus due to grooming and rubbing of the lesion following scarification cannot be discounted as means of transferring virus from the scarification site to these other external skin sites. There was no strong evidence of viral replication in tissues other than the scarification site that would support a systemic spread of the virus. This is different to what was found in naturally infected red squirrels and for other poxvirus infections (SQFV) of grey squirrels in America (Terrel *et al*., 2002; Bangari *et al*., 2009; King *et al*., 1972).

Although it was not possible to confirm virus replication in most of the SQPV DNA positive samples, nor the presence of infectious virus particles, it was interesting to observe that viral DNA was still detectable six weeks after initial inoculation suggesting that long term shedding of the virus by the grey squirrel might be a possibility. Viral DNA was also detected in the animals that had been re-challenged with the virus and sampled 3 weeks later, suggesting that the virus may be able to persist in animals despite their being exposed to the virus on previous occasions.

The grey squirrels sero-converted after being infected with SQPV via scarification, but only after a second challenge. Tomkins *et al.* (2002) also reported that sero-conversion of experimentally infected grey squirrels was not convincing until they were re-challenged. This contrasts with the ELISA readings obtained from naturally infected grey squirrels, where antibody titres are generally far greater (in terms of OD_{450}) than those obtained from the experimental infections. This may point to several possible explanations. It may be that the experimentally infected animals received a lower dose of virus or the route of inoculation was different from that to which animals in the wild would be subjected. Alternatively, it may suggest that grey squirrels in the wild are being exposed to the virus much more frequently if not constantly.

Red squirrels too appear to differ in their antibody response to infection with SQPV. Within the naturally infected red squirrels, which were found to be qPCR positive for SQPV, 50% did not have circulating antibodies against the virus. This is thought to be due to the animals dying before sero-conversion, suggesting a rapid progression of disease. This is despite some of the red squirrels having very high viral loads in skin

lesions. The high rate of morbidity and mortality in naturally infected red squirrels and the low sero-prevalence of antibodies to SQPV has led researchers to suggest that the red squirrel is not the natural host species for SQPV since the vast majority of infected animals die without any signs of recovery (Carroll *et al*., 2009; Sainsbury *et al*., 2000; Sainsbury *et al*., 2008; Tompkins *et al*., 2002).

It is impossible to know if the animals with detectable antibody responses had been exposed to the virus previously (hence the detectable antibody response) and survived only to succumb to a subsequent exposure, or if they had just survived longer than usual and long enough for their antibody response to be detected. Either way it might suggest that antibodies against the virus are insufficient to protect red squirrels from the lethal consequences of infection. Carroll and colleagues (Carroll *et al*., 2009), presented a model of host-pathogen dynamics where young red squirrels in particular are more susceptible to SQPV disease. However, in both this thesis and work described by Sainsbury *et al.* (2008) it was shown that some red squirrels did produce antibodies to the virus. Some of these animals still succumb to disease and die, whilst others appear to have survived the infection (Sainsbury *et al*., 2008).

Parallels can be drawn with the history of the myxoma virus (MYXV) within its natural hosts (*Sylvilagus* genus), and its consequences for the naïve population of European rabbits (*Oryctolagus cuniculus*) may provide hope for the red squirrel. In Australia, where MYXV was intentionally released, within 50 years the virus and rabbits appeared to co-evolve to a position whereby a less virulent MYXV and a population of rabbits less susceptible to the lethal manifestation of disease were selected (Spiesschaert *et al*., 2011). The result is that the wild population of rabbits now surviving in Australia do so in the presence of myxomatosis. It has been suggested, however, that this situation arose only because there were no natural reservoirs of MYXV in Australia. The situation in the UK with squirrelpox is different. The natural host, the grey squirrel, presumably has already undergone a considerable period of co-evolution with the virus in the USA before the virus was imported to the UK and encountered the naïve red squirrels. Since its natural host is present in considerable numbers in the UK there is presumably less evolutionary pressure for the virus to evolve from a position in which it causes an apparently benign infection in the grey squirrel. It is far too early to predict whether the animals

reported by Sainsbury *et al*., (2008) to have survived infection are the beginnings of a population that have natural resistance to the virus or have evolved resistance to the disease.

Within the UK in recent years, red squirrel captive-breeding and release programmes, designed to bolster populations in parts of the UK that have suffered the most dramatic losses of their red squirrels, have been undertaken. Translocations of red squirrels from healthy, potentially sustainable, populations have also been attempted. Unfortunately these reintroductions have seldom succeeded as often the red squirrels quickly succumbed to SQPV infection (Sainsbury & Gurnell 1995; Carroll *et al*., 2009). It was partly for this reason that research into the potential for producing a vaccine to protect vulnerable red squirrel populations was proposed.

The work described here (**Chapter 6**) for the first time confirmed that the disease being diagnosed across the country as squirrelpox had a single aetiological agent, namely SQPV. Before this, sequence data had been obtained from only one isolate of the virus, whereas now data has been obtained from seven isolates. The limited analysis that was possible indicates little variation in the viral genomic sequence and suggests that if vaccination is possible strain variation between the viruses present in the UK is unlikely to be a complicating factor.

Recent emphasis has drawn attention to the role of infectious disease as a major threat to endangered species and the consequences this has on biodiversity losses (Woolhouse *et al*., 2009). The situation with squirrelpox and red squirrels in the UK is just one other example of how a disease (caused by a micro or macro parasite), can have disruptive effect on the equilibrium of small, endangered populations. Other examples include the introduction of rinderpest to Africa which caused a drastic decline of the buffalo population in Kenya (Daszak & Cunningham 2000); rabies virus in wild dogs (*Lycaon pictus*) , again in Africa, has resulted in the decline of Ethiopian wolves (*Canis simensis*) (Cleaveland *et al*., 2001); the spread of distemper virus has had serious effects on black-footed ferrets (*Mustela nigripes*) (Williams *et al*., 1988), on the Northern Sea Otters of the Pacific ocean (Goldstein *et al*., 2009) and on Common Seal populations in Northern Europe (Harkonen *et al*., 2006); and protozoan infections, such as malaria, have affected Hawaiian avifauna (Feldman *et*

al., 1995). The worldwide animal trade (whether legal or illegal) and introductions of non-native species are increasing on a global scale with the inherent risk of introducing infectious diseases to immunologically naïve hosts. Hence, the control of invasive species and prohibition of animal translocations to new geographic regions represents one of the major challenges for maintenance of biodiversity, public health and socio-economic stability (Rhyan & Spraker, 2010).

7.2 Concluding remarks

The results of this thesis suggest that SQPV infection in grey squirrels is subclinical and the virus may be acquired by inoculation through damaged skin via fomites or via direct contact with infected animals. Once a grey squirrel has contracted the infection it is possible that virus will be shed from the mild scab that forms at the site of infection, therefore contaminating the environment (e.g. dreys, feeding stations and scent marking sites) and perpetuating the cycle of virus transmission. In contrast, once the infection spills over into the naïve red squirrel population, disease characterized by exudative lesions and proliferative scab formation, becomes apparent. The lesions carried by red squirrels are likely then to become the major source of infectious virus particles in the local environment and these obviously can be shed in the same way as suggested for the grey squirrels. In addition, considering the very high concentration of virus in scab and skin samples of squirrelpox affected red squirrels, the importance of mechanical transmission of the virus by ectoparasite should not be underestimated even though their epidemiological role in sustaining disease is not proven.

Red squirrels in particular, are regarded as a cryptic species, spending much of their time in the tree canopy and therefore it is thought only a small percentage of the population are encountered by conservation workers. Detection of epidemic disease in wildlife can be very difficult and assessing the risk factors for diseases in which conservation programmes are a matter of urgency, is a very challenging task which depends on several variables such as ecological interaction between animals, habitat structures and species densities (Wolfe *et al*., 2007). Therefore robust surveillance strategies and early diagnosis of infection are fundamental to stop the spread of

disease. The current methods of detecting SQPV infection in red squirrels depend on animals that are already exhibiting signs of disease and therefore diagnosis of an outbreak may lag behind what is happening in the field. The qPCR assay described here could be a useful addition to the diagnostic repertoire as it can detect subclinically infected animals, whilst this is unlikely to be of direct use in red squirrels, viral DNA was detectable in grey squirrels that were regarded as sero-negative using the 0.2 cut off point for the ELISA. Thus if finances permitted, surveillance programmes for detecting SQPV in the grey squirrel population could include qPCR analysis of eyelid skin as an earlier means of detecting of SQPV infection.

There are many questions about squirrelpox left unanswered after this study, partly due to lack of resources and limited access to animals, but especially due to the inability to perform an experimental infection in red squirrels and the practical and ethical difficulties associated with infection models in wild animals and endangered species. Further research is required to confirm that both experimentally SQPV infected grey squirrels and seropositive grey squirrels are capable of transmitting the virus to naïve red squirrels. Research should be undertaken to confirm whether transmission requires direct contact between animals or if SQPV infection can be contracted by the red squirrels through environmental contamination. The effects of SQPV infection on grey squirrels could be investigated further to see, for example, if subtle effects on longevity or fecundity, as demonstrated for cowpox in bank voles (Feore *et al*., 1997), are present.

Finally, the cause of death of SQPV infected red squirrels should be investigated; scab formation and skin ulcerations, with the absence of any manifested gross and histopathological lesions in the internal organs are unlikely to be the main reason for death. Several alternative hypotheses have been suggested, for example starvation, blindness and stress, but none of them have been confirmed. An explanation might be that the skin lesions make the animal more susceptible to secondary bacterial infections with death the result of septicaemia. Another alternative could be that the results of virus/host immune response interaction may lead to an uncontrolled and fulminant systemic inflammation (Stanford *et al*., 2007), with important changes in

squirrel physiology and behaviour. However as yet there is no evidence that either of these scenarios are the likely explanation.

The UK is still dealing with the consequences of grey squirrels 130 years after they were first introduced and 80 years after their translocation and release within the UK was made illegal by the 1934 Wildlife and Countryside Act. Although the work reported here has increased our understanding of SQPV in both red and grey squirrels it is difficult to know whether or not we are any nearer a solution for the red squirrels.

REFERENCES

Alcami, A. (2003). Viral mimicry of cytokines, chemokines and their receptors. *Nature Reviews Immunology,* **3**, 36-50.

Altshul, S., Gish, W., Miller, W., Myers, E. & Lipman, D. (1990). Basic local alignment search tool. *Journal of Molecular Biology,* **215**, 403-410.

Anon. Saving Scotland's red squirrels: project plan. (2009). Edinburgh; UK, Scottish Wildlife Trust.
Ref Type: Report

Arita, I., Gispen, R., Kalter, S.S., Wah, L. T., Marennikova, S. S., Netter, R. & Tagaya, I. (1972). Outbreaks of monkeypox and serological surveys in nonhuman primates. *Bulletin of the World Health Organization,* **46**, 625-631.

Arita, I. & Henderson, D.A. (1968). Smallpox and monkeypox in non-human primates. *Bulletin of the World Health Organization,* **39**, 277-283.

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

Bangari, D.S., Miller, M.A., Stevenson, G.W., Thacker, H.L., Sharma, A. & Mittal.S (2009). Cutaneous and Systemic Poxviral Disease in Red (*Tamiasciurus hudsonicus*) and Gray (*Sciurus carolinensis*) squirrels. *Veterinary Pathology,* **46**, 667-672.

Baroudy, B.M. & Moss, B. (1982). Sequence homologies of diverse length tandem repetitions near ends of vaccinia virus genome suggest unequal crossing over. *Nucleic Acids Research,* **10,** 5673-5679.

Baxby, D. (1977a). Is cowpox misnamed? A review of 10 human cases. *British Medical Journal,* **1**, 1379-1381.

Baxby, D. (1977b). Poxvirus Host and Reservoirs. *Archives of Virology,* **55,** 169-179.

Baxby, D., Ashton, D. G., Jones, D. M. & Thomsett, L. R. (1982). An outbreak of cowpox in captive cheetahs: virological and epidemiological studies. *The Journal of Hygiene (Lond),* **89**, 365-372.

Baxby, D., Shackleton, W.B., Wheeler, J. & Turner, A. (1979). Comparison of cowpox-like viruses isolated from European zoos. Brief report. *Archives of Virology,* **61**, 337-340.

Bengali, Z., Townsley, A.C. & Moss, B. (2009). Vaccinia virus strain differences in cell attachment and entry. *Virology,* **389**, 132-140.

Bennett, M. & Baxby, D. (1996). Cowpox. *Journal of Medical Microbiology,* **45**, 157-158.

Bennett, M., Gaskell, C.J., Gaskell, R.M., Baxby, D. & Gruffydd-Jones, T.J. (1986). Poxvirus infection in the domestic cat: some clinical and epidemiological observations. *Veterinary Record,* **118**, 387-390.

Bowden, T.R., Babiuk, S.L., Parkyn, G.R., Copps, J.S. & Boyle, D.B. (2008). Capripoxvirus tissue tropism and shedding: A quantitative study in experimentally infected sheep and goats. *Virology,* **371**, 380-393.

Broyles, S.S., Liu, X., Zhu, M. & Kremer, M. (1999). Transcription factor YY1 is a vaccinia virus late promoter activator. *The Journal of Biological Chemistry,* **274**, 35662-35667.

Bruemmer, C.M., Rushton, S.P., Gurnell, J., Lurz, P.W., Nettleton, P., Sainsbury, A.W., Duff, J.P., Gilray, J. & McInnes, C.J. (2010). Epidemiology of squirrelpox virus in grey squirrels in the UK. *Epidemiology and Infection,* **138**, 941-950.

Buist J.B. (1886) The life-history of the micro-organisms associated with variola and vaccina. *Proceeding of the Royal Society of Edinburgh* **13**, 603-620.

Buller, R.M. I. & Palumbo, G.J. (1991). Poxvirus Pathogenesis. *Microbiological Reviews,* **55**, 80-122.

Burley, J.R.W. (1986). Advances in the integreted control of the European rabbit in South Australia. *Proceedings of the 12th Vertebrate Pest Conference*. **12**, 140-146

Burton, J. L. (1994). Of mice and milkmaids, cats and cowpox. *Lancet,* **343**, 67.

Campe, H., Zimmermann, P., Glos, K., Bayer, M., Bergemann, H., Dreweck, C., Graf, P., Weber, B.K., Meyer, H., Buttner, M., Busch, U. & Sing, A. (2009). Cowpox virus transmission from pet rats to humans, Germany. *Emerging Infectious Diseases,* **15**, 777-780.

Carlton, M.H. & Reilly, J.R. (1955). Skin Tumors on Squirrels. *The Journal of Wildlife Management,* **19**, 402-403.

Carroll, B., Russell, P., Gurnell, J., Nettleton, P. & Sainsbury, A.W. (2009). Epidemics of squirrelpox virus disease in red squirrels (*Sciurus vulgaris*): temporal and serological finding. *Epidemiology and Infection,* **137**, 257-265.

(CDC) Centers for Disease Control and Prevention. update: multistate outbreak of monkeypox-Illinois, Indiana, Kansas, Missouri, Ohio, and Wisconsin, 2003.(2003) Morbidity and MortalityWeekly Report **52** (27), 642-646.
Ref Type: Report

Chantrey, J., Meyer, H., Baxby, D., Begon, M., Bown, K.J., Hazel, S.M., Jones, T., Montgomery, W.I. & Bennett, M. (1999). Cowpox: reservoir hosts and geographic range. *Epidemiology and Infection,* **122**, 455-460.

Cleaveland, S., Laurenson, M.K. & Taylor, L.H. (2001). Diseases of humans and their domestic mammals: Pathogen characteristics, host range and the risk of emergence. *Philosophical Transaction of the Royal Society of London series:B,* **356**, 991-999.

Coras, B., Essbauer, S., Pfeffer, M., Meyer, H., Schroder, J., Stolz, W., Landthaler, M. & Vogt, T. (2005). Cowpox and a cat. *Lancet,* **365**, 446.

Currado, I. (1998). The grey squirrel (*Sciurus carolinensis* Gmelin) in Italy: a potential problem for the entire European continent. In Steel M.A, Merritt J.F & Zegers D.A (Eds) *Ecology and Evolutionary Biology of the Tree squirrels* (pp. 263-266). Virginia Museum of Natural History, Special Publication, no.6.

Dagleish, P.M. & McInnes, C.J. (2011). Personal Communication.

Daszak, P. & Cunningham, A. A. (2000). Emerging Infectious Diseases of Wildlife--Threats to Biodiversity and Human Health. *Science,* **287**, 443.

David-Gray, Z.K., Gurnell, J. & Hunt, D.M. (1998). DNA fingerprinting reveals high levels of genetic diversity within British populations of the introduced non-native grey squirrel (*Sciurus carolinensis*). *Journal of Zoology*, **246**, 443-445.

De las Heras, M., Ortin, A., Salvatori, D., Perez, de V.M., Cousens, C., Miguel, F.L., Miguel, C.L., Garcia de Jalon, J.A., Gonzalez, L. & Michael, S.J. (2005). A PCR technique for the detection of Jaagsiekte sheep retrovirus in the blood suitable for the screening of ovine pulmonary adenocarcinoma in field conditions. *Research in Veterinary Science,* **79**, 259-264.

DiGiacomo, R.F. & Mare', C.J. (1994). Viral diseases. In Manning, P.J., Ringler, D.H. & Newcomer, C.E. (Eds) *The biology of the Laboratory Rabbit* (pp. 171-183). San Diego, California: Academic Press, Inc.

Downie, A.W. & Dumbell, K.R. (1947). Survival of variola virus in dried exudate and crusts from smallpox patients. *Lancet,* **1**, 550-553.

Downie, A.W. & Dumbell , K.R. (1956). Pox viruses. *Annual Review of Microbiology,* **10**, 237-252.

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

Edwards, F.B. (1962). Red Squirrel disease. *Veterinary Record,* **74**, 739-741.

Eis-Hubinger, A.M., Gerritzen, A., Schneweis, K.E., Pfeiff, B., Pullmann, H., Mayr, A. & Czerny, C.P. (1990). Fatal cowpox-like virus infection transmitted by cat. *Lancet,* **336**, 880.

Essbauer, S., Meyer, H., Porsch-Ozcurumez, M. & Pfeffer, M. (2007). Long-lasting stability of vaccinia virus (orthopoxvirus) in food and environmental samples. *Zoonoses and Public Health,* **54**, 118-124.

Essbauer, S., Pfeffer, M. & Meyer, H. (2010). Zoonotic poxviruses. *Veterinary Microbiology,* **140**, 229-236.

Esteban, D.J. & Buller, R.M.L. (2005). Ectromelia virus: The causative agent of mousepox. *Journal of General Virology,* **86**, 2645-2659.

Esteban, D. & Hutchinson, A. (2011). Genes in the terminal regions of orthopoxvirus genomes experience adaptive molecular evolution. *BMC Genomics,* **12**, 261.

Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U. & Ball, L.A. (eds) 2005. *Virus Taxonomy*. VIIIth Report of the International Committee on taxonomy of Viruses (ICTV) (pp 117-133). Elsevier: Academic Press, London.

Feldman, R.A., Freed, L.A. & Cann, R.L. (1995). A PCR test for avian malaria in Hawaiian birds. *Molecular Ecology,* **4,** 663-673.

Fenner, F. (1949). Mouse-Pox (Infectious Ectromelia of Mice): A Review. *The Journal of Immunology,* **63**, 341-373.

Fenner, F. (1965). Viruses of the myxoma-fibroma subgroup of the poxviruses. II. Comparison of soluble antigens by gel diffusion tests, and a general discussion of the subgroup. *Australian Journal of Experimental Biology & Medical Science,* **43**, 143-156.

Fenner, F. (1994). Monkeypoxvirus. Tetrapoxvirus. In A.D.M.E. Osterhaus (Ed). *Virus Infection of Rodents and Lagomorphs* (pp. 33-38). Amsterdam: Elsevier Science.

Fenner, F. (2000). Adventures with poxviruses of vertebrates. *FEMS Microbiology Reviews,* **24**, 123-133.

Fenner, F., Ratcliffe, F.N. (1965). Myxomatosis. Cambridge University Press. Cambridge, England.

Feore, S.M., Bennett, M., Chantrey, J., Jones, T., Baxby, D. & Begon, M. (1997). The effect of cowpox virus infection on fecundity in bank voles and wood mice. *Proceedings of Biological Science,* **264**, 1457-1461.

Goldstein, T., Mazet, J.A., Gill, V.A., Doroff, A.M., Burek, K.A. & Hammond, J.A. (2009). Phocine distemper virus in northern sea otters in the Pacific Ocean, Alaska, USA. *Emerging Infectious Diseases,* **15**, 925-927.

Greenwood, A. (2003). Pox disease in all taxa. In Fowler, M.E. & Miller, R.E. (Eds) *Zoo and Wild Animal Medicine* (pp. 737-741). St. Louis, MO: Saunders.

Guarner, J., Johnson, B.J., Paddock, C.D., Shieh, W.J., Goldsmith, C.S., Reynolds, M. G., Damon, I.K., Regnery, R.L. & Zaki, S.R. (2004). Monkeypox transmission and pathogenesis in prairie dogs. *Emerging Infecioust Disease,* **10**, 426-431.

Gubser, C., Hue, S., Kellam, P. & Smith, G.L. (2004). Poxvirus genomes: a phylogenetic analysis. *Journal of General Virology,* **85**, 105-117.

Gurnell, J. (1987). *The Natural History of Squirrels*. London: Christopher Helm.

Gurnell, J. & Hare, E.J. (2008). Rodents: Order Rodentia. In Harris, S. & Yalden, D. W. (Eds) *Mammals of the British Isles* (pp. 54-72). The Mammal Society.

Gurnell, J., Rushton, S.P., Lurz, P.W.W., Sainsbury, A.W., Nettleton, P., Shirley, M.D. F., Brummer C. & Geddes, N. (2006). Squirrel poxvirus: Landscape scale strategies for managing disease threat. *Biological Conservation,* **131**, 287-295.

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

Haig, D.M. (2001). Subversion and piracy: DNA viruses and immune evasion. *Research in Veterinary Science,* **70**, 205-219.

Hale, M.L. & Lurz, P.W.W. (2003). Morphological changes in a British mammal as a result of introductions and changes in landscape management: the red squirrel (*Sciurus vulgaris*). *Journal of Zoology,* **260**, 159-167.

Harkonen, T., Dietz, R., Reijnders, P., Teilmann, J., Harding, K., Hall, A., Brasseur, S., Siebert, U., Goodman, S. J., Jepson, P.D., Dau, R.T. & Thompson, P. (2006). The 1988 and 2002 phocine distemper virus epidemics in European harbour seals. *Diseases of Aquatic Organisms,* **68,** 115-130.

Harvie-Brown, J.A. (1878-1881). The history of the squirrel in Great Britain. *Proceedings of the Royal Physical Society of Edinburgh,* **5**: 343-48; **6**: 31-63, 115-83.

Hawranek, T., Tritscher, M., Muss, W.H., Jecel, J., Nowotny, N., Kolodziejek, J., Emberger, M., Schaeppi, H. & Hintner, H. (2003). Feline orthopoxvirus infection transmitted from cat to human. *Journal of the American Academy of Dermatology,* **49**, 513-518.

Hazel, S.M., Bennett, M., Chantrey, J., Bown, K., Cavanagh, R., Jones, T.R., Baxby, D. & Begon, M. (2000). A longitudinal study of an endemic disease in its wildlife reservoir: cowpox and wild rodents. *Epidemiology and Infection,* **124**, 551-562.

Hemmer, C.J., Littmann, M., Löbermann, M., Meyer, H., Petschaelis, A. & Reisinger, E. C. (2010). Human cowpox virus infection acquired from a circus elephant in Germany. *International Journal of Infectious Diseases,* **14**, e338-e340.

Himsworth, C.G., Musil, K.M., Bryan, L. & Hill, J.E. (2009). Poxvirus Infection in an American Red squirrel (Tamiasciurus hudsonicus) from Northwestern canada. *Journal of Wildlife Diseases,* **45**, 1143-1149.

Hirth, R.S., Wyand, D.S., Osborne, A.D. & Burke, C.N. (1969). Epidermal changes caused by squirrel poxvirus. *Journal of American Veterinary Medical Association,* **155**, 1120-1125.

Holland, M.P., Palmarini, M., García-Goti, M., Gonzales, L., McKendrick, I., De las Heras, M., Sharp, J.M. (1999). Jaagsiekte retrovirus is widely distributed both in T and in B lymphocytes and in mononuclear phagoocytes of sheep with naturally and experimentally acquired pulmonary adenomatosis. *Journal of Virology,* **73**, 6964-6972.

Hughes, A.L., Irausquin, S. & Friedman, R. (2010). The evolutionary biology of poxviruses. *Infection, Genetics and Evoution.* **10**, 50-59.

Hutson, C.L., Olson, V.A., Carrol, D.S., Abel, J.A., Hughes, C.M., Braden, Z.H., Weiss, S., Self, J., Osorio, J.E., Hudson, P.N., Dillon, M., Karem, K.L., Damon, I.K. & Regnery, R.L. (2009). A prairie dog animal model of systemic arthopoxvirus disease using West African and Congo Basin strain of monkeypox virus. *Journal of General Virology,* **90**, 323-333.

Joklik, W.K. & Becker, Y. (1964). The replication and coating of Vaccinia DNA. *Journal of Moecular Biology,* **10**, 452-474.

Kato, S. & Cutting, W. (1959). A study of the inclusion bodies of rabbit myxoma and fibroma virus and a consideration of the relationship between all pox virus inclusion bodies. *Stanford Medical Bulletin,* **17**, 34-45.

Kaysser, P., von Bomhard, W., Dobrzykowski, L. & Meyer, H. (2010). Genetic diversity of feline cowpox virus, Germany 2000-2008. *Veterinary Microbiology,* **141**, 282-288.

Kenward, R.E. & Holm, J.L. (1993). On the replacement of the red squirrel in Britain: a phytotoxic explanation. *Proceedings of Royal Society of London.B,* **251**, 187-194.

Kerr, P. & McFadden, G. (2002). Immune responses to myxoma virus. *Viral Immunology,* **15**, 229-246.

Keymer, I. F. (1974). Report of the pathologist, 1971 and 1972. The Zoological Society of London, Scientific Report 1971-1973. *Journal of Zoology, London,* **173**, 55.

Keymer, I.F. (1983). Diseases of squirrels in Britain. *Mammal Review,* **13**, 155-158.

Khodakevich, L., Jezek, Z. & Kinzanzka, K. (1986). Islation of Monekeypox virus from wild squirrel infected in nature. *The Lancet,* **327**, 98-99.

Khodakevich, L., Jezek, Z. & Messinger, D. (1988). Monkeypox virus: ecology and public health significance. *Bulletin of World Health Organization,* **66**, 747-752.

Khodakevich, L., Szczeniowski, M., Manbu-ma. D., Jezek, Z., Marennikova, S., Nakano, J. & Messinger, D. (1987). The role of squirrels in sustaining monkeypox virus transmission. *Tropical and Geographical Medicine,* **39**, 115-122.

Kilham, L. & Dalmat, H.T. (1955). Host-virus-mosquito relations of Shope fibromas in cottontail rabbits. *American Journal of Hygiene,* **61,** 45-54.

Kilham, L., Herman, C. M. & Fisher, E. R. (1953). Naturally occurring fibromas of grey squirrels related to Shope's rabbit fibroma. *Proceeding of Society of Experimental Biology and Medicine,* **82**, 298-301.

King, J.M., Wolf, A. & Shively, J.N. (1972). Naturally occurring squirrel fibroma with involvement of internal organs. *Journal of Wildlife Diseases,* **8**, 321-324.

Kirschstein, R.L., Rabson, A.S. & Kilham, L. (1958). Pulmonary lesions produced by fibroma viruses in squirrels and rabbits. *Cancer Research,* **18**, 1340-1344.

Koprowski, J.L. (1994). Sciurus carolinensis. *Mammalian Species,* **480**:1-9.

Kurth, A., Straube, M., Kuczka, A., Dunsche, A.J., Meyer, H. & Nitsche, A. (2009). Cowpox virus outbreak in banded mongooses (Mungos mungo) and jaguarundis (Herpailurus yagouaroundi) with a time-delayed infection to humans. *PLoS ONE,* **4**, e6883.

Langohr, I.M., Stevenson, G.W., Thacker, H.L. & Regnery, R.L. (2004). Extensive Lesions of Monkeypox in a Prairie Dog (Cynomys sp.). *Veterinary Pathology Online*, **41**, 702-707.

LaRose, J.P., Meredith, A.L., Everest, D.J., Fiegna, C., McInnes, C.J., Shaw, D.J. & Milne, E.M. (2010). Epidemiological and postmortem findings in 262 red squirrels (Sciurus vulgaris) in Scotland, 2005 to 2009. *Veterinary Record,* **167**, 297-302.

Lawn, R. (2010). Risk of cowpox to small animal practitioners. *Veterinary Record,* **166**, 631.

Lefkowitz, E.J., Wang, C. & Upton, C. (2006). Poxviruses: past, present and future. *Virus Research,* **117**, 105-118.

Lloyd, H.G. (1983). Past and present distribution of Red and Grey squirrels. *Mammal Review*, **13**, 69-80.

Macdonald, D., Burnham, D. & WildCRU . *The state of Britain's Mammals 2011*. People's trust for endagered species. Ninth annual report (ed) (2011). The University of Oxford, Department of Zoology's Wildlife Conservation Research Unit (WILDCRU).

Mahy, B.W.J. & Kangro, H.O. (1996). *Virology Methods Manual*. San Diego, USA: Academic Press.

Marennikova, S. S., Maltseva, N.N., Korneeva, V.I. & Garanina, N. (1977). Outbreak of pox disease among carnivora (felidae) and edentata. *Journal of Infectious Diseases,* **135**, 358-366.

Marennikova, S. S., Shelukhina, E. M. & Efremova, E. V. (1984). New outlook on the biology of cowpox virus. *Acta Virology,* **28**, 437-444.

Martland, M.F., Poulton, G.J. & Done, R.A. (1985). Three cases of cowpox infection of domestic cats. *Veterinary Record,* **117**, 231-233.

McFadden, G. (2005). Poxvirus tropism. *Nature Reviews Microbiology,* **3,** 201-213.

McInnes, C.J., Coulter, L., Dagleish, M.P., Fiegna, C., Gilray, J., Willoughby, K., Cole, M., Milne, E., Meredith, A., Everest, D.J. & MacMaster, A.M. (2009). First cases of squirrelpox in red squirrels (*Sciurus vulgaris*) in Scotland. *Veterinary Record,* **164**, 528-531.

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

Mercer, A.A., Ueda, N., Friederichs, S.M., Hofmann, K., Fraser, K.M., Bateman, T. & Fleming, S.B. (2006). Comparative analysis of genome sequences of three isolates of Orf virus reveals unexpected sequence variation. *Virus Research,* **116**, 146-158.

Mercer, J. & Helenius, A. (2008). Vaccinia virus uses macropinocytosis and apoptotic mimicry to enter host cells. *Science,* **320**, 531-535.

Middleton, A.D. (1930) The Ecology of the American Grey Squirrel (*Sciurus carolinensis* Gmelin) in the British Isles. *Proceedings of the Zoological Society London,* part 3: 809-843

Middleton, A.D. (1931). *The Grey squirrel.* London: Sidgwick & Jackson.

Moss, B. (2007). Poxviridae: The Viruses and Their Replication. Fifth edition. In D. M. Knipe & P. M. Howley (Eds) *Fields virology* (pp. 2905-2947). Philadelphia: Lippincott Williams & Wilkins.

Murphy, F.A. (1999). Poxviridae. *Veterinary Virology* (pp. 278-291). London: Academic Press.

Nitsche, A., Kurth, A. & Pauli, G. (2007). Viremia in human Cowpox virus infection. *Journal of Clinical Virology*, **40**, 160-162.

O'Connor, D.J., Diters, R.W. & Nielsen, S.W. (1980). Poxvirus and multiple tumors in an eastern gray squirrel. *Journal of the American Veterinary Medical Association.* **177**, 792-795.

Parrott, D., Quy, R., Driel, V.K., Lurz, P., Rushton, S., Gurnell, J., Aebischer, N. & Reynolds, J. (2009). *Review of red squirrel conservation activity in Northern England*. Natural England Commissioned Report NECR019. Sheffield: Natural England.

Peacock, D.E. (2009). The Grey Squirrel Sciurus Carolinensis in Adelaide, South Australia: Its Introduction and Eradication . *The Victoriam Naturalist,* **126**, 150-156.

Pfeffer, M., Pfleghaar, S., von, B.D., Kaaden, O.R. & Meyer, H. (2002). Retrospective investigation of feline cowpox in Germany. *Veterinary Record,* **150**, 50-51.

Postma, B. H., Diepersloot, R.J., Niessen, G.J. & Droog, R.P. (1991). Cowpox-virus-like infection associated with rat bite. *Lancet,* **337**, 733-734.

Rasmussen, R. (2001). Quantification on the LightCycler. In Meuer, S., Wittwer C. & Nakagawara, K. (Eds) *Rapid Cycle Real-Time PCR, Methods and applications* (pp. 21-34). Heidelberg: Springer Press.

Ratcliff, R.M., Chang, G., Kok, T. & Sloots, T.P. (2007). Molecular diagnosis of medical viruses. *Current Issues in Molecular Biology,* **9,** 87-102.

Regnery, R.L. (1975). Preliminary Studies on an Unusual Poxvirus of the Western Grey squirrel (*Sciurus griseus griseus*) of North America. *Intervirology,* **5**, 364-366.

Reynolds, M.G., Carroll, D.S., Olson, V.A., Hughes, C., Galley, J., Likos, A., Montgomery, J.M., Suu-Ire, R., Kwasi, M.O., Jeffrey Root, J., Braden, Z., Abel, J., Clemmons, C., Regnery, R., Karem, K. & Damon, I.K. (2010). A Silent Enzootic of an Orthopoxvirus in Ghana, West Africa: Evidence for Multi-Species Involvement in the Absence of Widespread Human Disease. *American Journal of Tropical Medicine and Hygiene,* **82**, 746-754.

Rhyan, J. C. & Spraker, T.R. (2010). Emergence of Diseases From Wildlife Reservoirs. *Veterinary Pathology Online,* **47**, 34-39.

Rutledge, R.G. & Côté, C. (2003). Mathematics of quantitative kinetic PCR and the application of standard curves. *Nucleic Acids Research,* **31**, e93.

Robert, B. T., Watts, D.M., Sbrana, E., Siirin, M., Vsevolod, L.P. & Xiao, S-Y. (2004). Experimental Infection of ground squirrels (*Spermophilus tridecemlineatus*) with Monkeypox Virus. *Emerging Infectious Diseases,* **10**, 1563-1567.

Roberts, K.L. & Smith, G.L. (2008). Vaccinia virus morphogenesis and dissemination. *Trends in Microbiology,* **16**, 472-479.

Robinson, A.J. & Kerr, P.J. (2001). Poxvirus Infections. In Williams, E.S. & Barker, I.K. (Eds) *Infectious Diseases Of Wild Mammals* (pp. 179-201). London: Manson.

Roingeard, P. (2008). Viral detection by electron microscopy: past, present and future. *Biol Cell,* **100**, 491-501.

Rushton, S.P., Lurz, P.W.W., Gurnell, J., Fuller, R. (2000). Modelling the spatial dynamics of parapoxvirus disease in red and grey squirrels: a possible cause of the decline in the red squirrel in the UK? *Journal of applied Ecology,* **37**, 997-1012

Rushton, S. P., Lurz, P.W., Gurnell, J., Nettleton, P., Bruemmer, C., Shirley, M.D. & Sainsbury, A.W. (2006). Disease threats posed by alien species: the role of a poxvirus in the decline of the native red squirrel in Britain. *Epidemiology and Infection,* **134**, 521-533.

Sainsbury, A.W. (2008). *The Epidemiology of Poxvirus Infection in Squirrels*. The Royal Veterinary College, Univerity of London.
Ref Type: Thesis/Dissertation

Sainsbury, A.W., Deaville, R., Lawson, B., Cooley, W.A., Farelly, S.S., Stack, M.J., Duff, P., McInnes, C.J., Gurnell, J., Russell, P.H., Rushton, S.P., Pfeiffer, D.U., Nettleton, P. & Lurz, P.W. (2008). Poxviral disease in red squirrels (*Sciurus vulgaris*) in the UK: spatial and temporal trends of an emerging threat. *EcoHealth,* **5**, 305-316.

Sainsbury, A.W. & Gurnell, J. (1995). An investigation into the health and welfare of red squirrels, *Sciurus vulgaris,* involved in reintroduction studies. *Veterinary Record,* **137**, 367-370.

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

Sainsbury, A.W., Nettleton, P. & Gurnell, J. (1997). Recent developments in the study of parapoxvirus in red and grey squirrels. In Gurnell, J. & Lurz, P.W.W. (Eds) *The Conservation of Red Squirrels, Sciurus vulgaris L.* (pp. 105-108). London: People's trust for endangered species.

Sainsbury, A.W. & Ward, L. (1996). Parapoxvirus infection in red squirrels. *Veterinary Record,* **138**, 400.

Sands J.J., Scott A.C. & Harkness J.W. (1984). Isolation in cell culture of a poxvirus from the red squirrel (Sciurus vulgaris). *Veterinary Record,* **114**, 117-118.

Sbrana, E., Xiao, S.Y., Newman, P.C. & Tesh, R.B. (2007). Comparative pathology of North American and central African strains of monkeypox virus in a ground squirrel model of the disease. *American Journal of Tropical Medicine and Hygiene,* **76**, 155-164.

Schmiedeknecht, G., Eickmann, M., Kohler, K., Herden, C.E., Kolesnikova, L., Forster, C., Burkhardt, E.H., Konig, M., Thiel, M. & Reinacher, M. (2010). Fatal cowpox virus infection in captive banded mongooses (Mungos mungo). *Veterinary Pathology,* **47**, 547-552.

Scola, B.L., Audic, S., Robert, C., Jungang, L., de Lamballerie, X., Drancourt, M., Birtles, R., Claverie, J.M. & Raoult, D. (2003). A Giant Virus in Amoebae. *Science*, **299,** 2033.

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

Scott, C.B., Holdbrook, R. & Sell, S. (1981b). Cell-mediated immune response to Shope fibroma virus-induced tumors in adult rabbits. *Journal of National Cancer Institute,* **66**, 681-689.

Seet, B.T., Johnston, J.B., Brunetti, C.R., Barrett, J.W., Everett, H., Cameron, C., Sypula, J., Nazarian, S.H., Lucas, A. & McFadden, G. (2003). Poxviruses and immune evasion. *Annual Review of Immunoogy,* **21**, 377-423.

Sell, S. & Scott, C.B. (1981). An immunohistologic study of Shope fibroma virus in rabbits: tumor rejection by cellular reaction in adults and progressive systemic reticuloendothelial infection in neonates. *Journal of National Cancer Institute,* **66**, 363-373.

Senkevich, T.G., Koonin, E.V., Bugert, J.J., Darai, G. & Moss, B. (1997). The genome of molluscum contagiosum virus: analysis and comparison with other poxviruses. *Virology*, **233**, 19-42.

Shiohara, T. & Mizukawa, Y. (2005). The immunological basis of lichenoid tissue reaction. *Autoimmunity Reviews*, **4**, 236-241.

Shope, R.E. (1932). A filtrable virus causing a tumor-like condition in rabbits and its relationship to virus Myxomatosum. *The Journal of Experimental Medicine,* **56**, 803-822

Shorten, M. (1954). *Squirrels*. London: Collins.

Simpson, V.R., Hargreaves, J., Everest, D.J., Baker, A.S., Booth, P.A., Butler, H.M. & Blackett, T. (2010). Mortality in red squirrels (Sciurus vulgaris) associated with exudative dermatitis. *Veterinary Record,* **167**, 59-63.

Smith, S.A. & Kotwal, G.J. (2002). Immune response to poxvirus infections in various animals. *Critical Reviews in Microbiology*, **28**, 149-185.

Spickler, A.R. & Roth, J.A. (2008). Descriptions of Recent Incursions of Exotic Diseases. Section 2- Facts sheets for Emerging and exotic Diseases of Animals. In Spickler, A.R. & Roth, J.A. (Eds) *Emerging and Exotic Diseases of Animals* (pp. 198-200). Iowa: Iowa State University.

Spiesschaert, B., McFadden, G., Hermans, K., Nauwynck, H. & Van de Walle, G.R. (2011). The current status and future directions of myxoma virus, a master in immune evasion. *Veterinary Research*, **42**, 76.

Stanford, M.M., McFadden, G., Karupiah, G. & Chaudhri, G. (2007). Immunopathogenesis of poxvirus infections: forecasting the impending storm. *Immunology and Cell biology*, **85**, 93-102.

Talkington, E. (2008) The seroepidemiology of Squirrelpox virus in the red squirrel (*Sciurus vulgaris*) and the grey squirrel (*Sciurus carolinensis*) of Great Britain (MSc Thesis). The Royal Veterinary College, University of London.
Ref Type: Thesis/Dissertation

Terrel, S.P., Forrester, D.J., Mederer, H. & Regan, T.W. (2002). An epizootic of fibromatosis in gray squirrels (*Sciurus carolinensis*) in Florida. *Journal of Wildlife Diseases*, **38**, 305-312.

Thomas, K., Tompkins, D.M., Sainsbury, A.W., Wood, A.R., Dalziel, R., Nettleton, P. F. & McInnes, C.J. (2003). A novel poxvirus lethal to red squirrels (*Sciurus vulgaris*). *Journal of General Virology*, **84**, 3337-3341.

Tompkins, D.M., Sainsbury, A.W., Nettleton, P., Buxton, D. & Gurnell, J. (2002). Parapoxvirus Causes a Deleterious Disease in Red Squirrels Associated with UK Population Declines. *Proceedings of the Royal Society of London series B: Biological Sciences*, **269**, 529-533.

Tompkins, D.M., White, A.R. & Boots, M. (2003). Ecological replacement of native red squirrels by invasive greys driven by disease. *Ecology letters*, **6**, 189-196.

Tryland, M., Sandvik, T., Mehl, R., Bennett, M., Traavik, T. & Olsvik, O. (1998). Serosurvey for orthopoxviruses in rodents and shrews from Norway. *Journal of Wildlife Diseases*, **34**, 240-250.

Tsanava, S. A., Sakvarelidze, L. A. & Shelukhina, E. M. (1989). Serologic survey of wild rodents in Georgia for antibodies to orthopoxviruses. *Acta Virology*, **33**, 91.

Upton, C., Slack, S., Hunter, A.L., Ehlers, A. & Roper, R.L. (2003). Poxvirus orthologous clusters: toward defining the minimum essential poxvirus genome. *The Journal of Viroogyl*, **77**, 7590-7600.

Vizoso, A.D. (1968). A red squirrel disease. *Symposium of the Zoological Society of London*, **24**, 29-38.

Vizoso, A.D. (1970). Tumor Viruses. *Proceedings of the Royal Society of Medicine*, **63**, 341-344.

von Bomhard, W., Mauldin, E.A., Breuer, W., Pfleghaar, S. & Nitsche, A. (2011). Localized cowpox infection in a 5-month-old Rottweiler. *Veterinary Dermatology*, **22**, 111-114.

Wallace, G.D. & Buller, R.M. (1985). Kinetics of ectromelia virus (mousepox) transmission and clinical response in C57BL/6j, BALB/cByj and AKR/J inbred mice. *Laboratory Animal Science*, **35,** 41-46.

Williams, E.S., Thorne, E.T., Appel, M.J. & Belitsky, D.W. (1988). Canine distemper in black-footed ferrets (Mustela nigripes) from Wyoming. *Journal of Wildlife.Diseases,* **24,** 385-398.

Wittek, R. & Moss, B. (1980). Tandem repeats within the inverted terminal repetition of vaccinia virus DNA. *Cell,* **21,** 277-284.

Wolfe, N.D., Dunavan, C.P. & Diamond, J. (2007). Origins of major human infectious diseases. *Nature,* **447**, 279-283.

Woolhouse, M.E.J., Haydon, D.T., & Antia, R. (2009). Emerging pathogens: the epidemiology and evolution of species jumps. *Trends in ecology and Evolution,* **20**, 238-244.

Xing, K., Deng, R., Wang, J., Feng, J., Huang, M. & Wang, X. (2006). Genome-based phylogeny of poxvirus. *Intervirology,* **49**, 207-214.

APPENDIX 1.0 - List of Reagents

Commonly used reagents

10 X Phosphate buffered saline (PBS), pH 7.4

1.37M sodium chloride, M.wt. 58.44 (Fisher Chemicals)	80 g
2.7mM potassium chloride, M.wt.141.96 (Sigma-Aldrich)	2 g
0.1M di-sodium hydrogen orthophosphate, M,wt. 141.96 (Sigma-Aldrich)	14.4 g
2 mM potassium phosphate monobasic, M.wt. 136.1 (Sigma-Aldrich)	2.4 g

Mix and adjust pH to 7.4. Make up the volume to 1 litre. Autoclave to sterilise.

0.5M Disodium ethylene diamine tetra-acetate (EDTA), pH 8

EDTA disodium salt, M.wt. 372.2 (Sigma-Aldrich)	186.1 g
Distilled water	400 ml

Mix and adjust pH to 8 with 10M NaOH. Make up the volume to 1 litre with distilled water. Autoclave to sterilise.

1M Tris-HCl, Ph variable

Tris base, M.wt. 121.1 (Promega)	121.1 g
Distilled water	800 ml

Mix and adjust pH to desired value with concentrated hydrochloric acid (HCl). Make up to 1 litre with distilled water. Autoclave to sterilise.

70% v/v ethanol

Molecular grade ethanol M.wt. 46.07 (Fisher Scientific)	70 ml
Distilled water	30 ml

95% v/v ethanol

Molecular grade ethanol M.wt. 46.07 (Fisher Scientific)	95 ml
Distilled water 30 ml	5 ml

AE buffer (pH 9) (QIAGEN®)

10mM Tris-HCl	10mM Tris-HCl
0.5 mM EDTA	0.5 mM EDTA

10 X TBE buffer (0.9M Tris Borate/20mM EDTA)

Tris base, M.wt. 121.1 (Promega)	108 g
Boric acid, M.wt. 61.83 (Sigma-Aldrich)	55 g
0.5M EDTA, pH 8	40ml
Distilled water	Up to 1000ml

6 X DNA loading buffer

Bromophenol blue sodium salt, M.wt. 691.9 (BDH)	0.025 g
Xylene cyanol FF, M.wt. 538.6 (Sigma-Aldrich)	0.025 g
Glycerol, M.wt. 92.09 (Sigma-Aldrich)	3 ml
Distilled water	7 ml

Tissue culture reagents

199 media base (200 ml)

10 X Medium 199 (Eagle's Medium; Sigma-Aldrich)	20 ml
29.5 g /litre Tryptose phosphate broth (TBP) (Moredun Scientific Services)	20 ml
80 g/litre sodium bicarbonate (Moredun Scientific Services)	4 ml
0.1M glutamine (Moredun Scientific Services)	4 ml
Distilled sterile water	132 ml

Growth 199 media

199 media base	180 ml
Foetal bovine sera (FBS) (GibcoBRL) heat inactivated at 56°C for 30 minutes	20 ml

Maintenance 199 media

199 media base	180 ml
Foetal bovine sera (FBS) (GibcoBRL) heat inactivated at 56°C for 30 minutes	4 ml
Distilled sterile water	16 ml

Molecular cloning

10% w/v 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal)

X-gal (Bioline)	100 mg
N,N-dimethylformamide (Sigma-Aldrich)	1 ml

2% w/v Isopropyl-ß-D-thio-galactopyranoside (IPTG)

IPTG (Bioline)	200 mg
Distilled water	10 ml

Filter through a 0.45 µm filter (Millipore) to sterilise.

Luira-Bertani broth (LB) (Moredun Scientific Services)

Bacto tryptone (DIFCO)	10 g
Bacto yeast extract (DIFCO)	5 g
Sodium chloride (Fisher Chemicals)	10 g
Distilled water	1000 ml

Mix and pH to 7.0, then autoclave to sterilise.

50mg/ml Ampicillin

Ampicillin (Sigma-Aldrich)	500 mg
Distilled water	10 ml

Mix then filter to sterilise

Enzyme-linked immunosorbent assay (ELISA) reagents

ELISA coating (carbonate-bicarbonate) buffer

0.2M sodium bicarbonate M.wt. 105.99 (Sigma-Aldrich)	7.9 ml
0.2M sodium hydrogen carbonate M.wt. 84.01(Sigma-Aldrich)	17.1 ml
Distilled water	75.0 ml
Correct the pH solution to 9.6 by the addition of 0.2M sodium bicarbonate or 0.2M sodium hydrogen carbonate.	

ELISA wash buffer

10 X phosphate buffer saline (PBS)	1000 ml
(Polyoxylethylene-sorbitan monolaurate) $Tween_{20}$ (Sigma-Aldrich)	5 ml
Distilled water	9000 ml

ELISA diluents buffer (PBS/0.05% v/v $Tween_{20}$/1% v/v BSA)

ELISA wash buffer	100 ml
Bovine serum albumin fraction V (Sigma-Aldrich)	1 g

Histology and Immunohistochemistry reagents

Scott's tap water substitute (STWS)

0.2M Sodium hydrogen carbonate (Sigma-Aldrich)	10 mg
Magnesium sulphate	10 mg
Distilled water	5 l

Antigen retrival (Tris-EDTA Buffer) (10mM Tris Base, 1mM EDTA Solution, 0.05% v/v $Tween_{20}$, pH 9.0)

Tris base, M.wt. 121.1 (Promega)	1.21 g
EDTA	0.37 g
Distilled water	1000 ml
Mix to dissolve and adjust pH, then add 0.5 ml of $Tween_{20}$	

10% v/v formal saline

Formaldehyde solution 40% v/v (Fisher Scientific)	500 ml

1.37M sodium chloride, M.wt. 58.44 (Fisher Chemicals)	42.5 g
Tap water	4.5 l

General use antibiotics and antimycotic and antibiotic

250 µg/ml Amphotericin B Fungizone® (Sigma-Aldrich)

10,000U/ml Penicillin, 10mg/ml Streptomycin solution (Moredun Scientific Services**)**

100U/ml Polymixin B Sulphate (Sigma-Aldrich**)**

APPENDIX 2.0 – Published Works

Papers

Epidemiological and postmortem findings in 262 red squirrels (*Sciurus vulgaris*) in Scotland, 2005 to 2009

J. P. LaRose, A. L. Meredith, D. J. Everest, C. Fiegna, C. J. McInnes, D. J. Shaw, E. M. Milne

Postmortem and virological examinations for squirrelpox virus (SQPV) were carried out on 262 red squirrels (*Sciurus vulgaris*) found dead or moribund in Scotland between September 2005 and July 2009, to determine the likely causes of death and highlight factors that might be threats to the red squirrel population. Most of the squirrels were submitted from Dumfries and Galloway, and 71 per cent of them were adults. Road traffic accidents, squirrelpox, trauma or starvation were responsible for death in a large proportion (73 per cent) of the squirrels. Thin or emaciated body condition was associated with deaths resulting from pneumonia SQPV infection and starvation, and with the presence of external parasites. There were differences between age groups with regard to the cause of death; a large proportion of juveniles died of starvation, whereas a large proportion of subadults and adults died in road traffic accidents. SQPV infection was associated with the presence of external parasites, but was not associated with the sex of the animals.

THE red squirrel (*Sciurus vulgaris*) is considered a vulnerable species in the UK and is included as a priority species in the UK government's conservation initiative, the UK Biodiversity Action Plan. Red squirrels are no longer present in many parts of England and Wales, and many of the existing populations are declining (Harris and others 1995). The published estimate of the size of the red squirrel population in Great Britain is 161,000 animals, comprising approximately 30,000 in England, 10,000 in Wales and 121,000 in Scotland; the red squirrels in Scotland therefore represent 70 to 75 per cent of the surviving population of the species in Great Britain (Harris and others 1995). The protection of this last remaining substantial red squirrel population is a priority if the species is not to become extinct on the UK mainland.

The red squirrel's demise has been attributed mainly to the presence of the American grey squirrel (*Sciurus carolinensis*), a non-native species first introduced into the UK in the late 19th century (Usher and others 1992, Kenward and Holm 1993). Direct competition between red and grey squirrels for resources may partly account for the decline in the red squirrel population, with grey squirrels tending to outcompete red squirrels in some types of habitat (Kenward and Holm 1993, Kenward and others 1998). However, the most significant factor in this relationship between the decline in the number of red squirrels and the presence of grey squirrels is now believed to be squirrelpox virus (SQPV) (Tompkins and others 2002, Rushton and others 2006). SQPV, which belongs to the Poxviridae subfamily Chordopoxviridae (McInnes and others 2006), infects grey squirrels with no apparent adverse effects on their health, but causes a severe, usually lethal, disease in red squirrels (Tompkins and others 2002). There is only one reported case of clinically evident naturally occurring SQPV disease in a grey squirrel (Duff and others 1996). In red squirrels, the disease is characterised by severe, erythematous, exudative dermatitis around the face, feet and ventrum. Ulceration and infection of these lesions, along with lethargy, may lead to the death of infected red squirrels in the wild (Tompkins and others 2002), although the existence of seropositive red squirrels suggests that some may recover (Sainsbury and others 2008). SQPV is blamed for the extinction of many red squirrel populations in England and Wales, and the grey squirrel is likely to be the reservoir host for the virus (Sainsbury and others 2008). Indirect evidence for the likely existence of SQPV in Scotland was not recorded until 2005, when grey squirrels seropositive for the virus were first identified there. Two years later, the first affected red squirrels were detected (McInnes and others 2009), demonstrating that SQPV now presents a serious threat to red squirrels in Scotland.

Although SQPV appears to have contributed significantly to the decline in the red squirrel population in Great Britain, it is not the only cause of death associated with this decline, especially in Scotland, where squirrelpox is not yet widespread. Other previously reported causes of mortality in red squirrels include predation, starvation, extreme cold weather, parasitic disease, failure to thrive after weaning, stress after relocation, neoplasia, nutritional disease and human activities (road traffic casualties) (Keymer 1983, Kenward and Hodder

Veterinary Record (2010) 167, 297-302 doi: 10.1136/vr.c4196

J. P. LaRose, BSc, BVM&S, MRCVS, **A. L. Meredith,** MA, VetMB, CertLAS, DZooMed, MRCVS, **D. J. Shaw,** BSc, PhD, **E. M. Milne,** BVM&S, PhD, DipECVCP, DipRCPath, FRCVS, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush Veterinary Centre, Roslin, Midlothian EH25 9RG
D. J. Everest, Veterinary Laboratories Agency – Weybridge, New Haw, Addlestone, Surrey KT15 3NB
C. Fiegna, DVM, **C. J. McInnes,** BSc, PhD, Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik, Midlothian EH26 0PZ

Dr Shaw and Professor Milne are also at The Roslin Institute, University of Edinburgh, Roslin, Midlothian EH25 9PS

Correspondence to Professor Milne, e-mail: elspeth.milne@ed.ac.uk

Provenance: not commissioned; externally peer reviewed

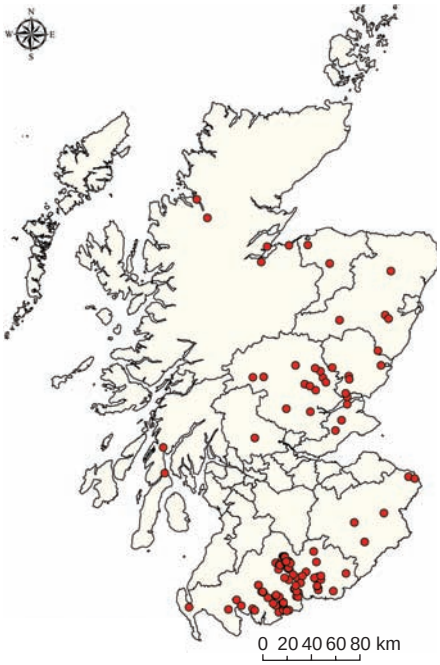


FIG 1: Grid reference number locations of 247 red squirrel carcases submitted from Scottish counties for postmortem examination between September 2005 and July 2009. Crown Copyright/database right 2009. An Ordnance Survey/EDINA supplied service

1998, Lurz and others 2005, Simpson and others 2006). There have also been occasional case studies on the causes of death and disease in individual red squirrels in the UK (Duff and others 2007, Simpson and others 2009). However, there has been no comprehensive study to examine and quantify the causes of death in a larger sample size of red squirrels in Scotland. The current threat to the red squirrel population in Scotland highlights the importance of gaining greater understanding of the factors responsible for mortality in this population. An ongoing scheme in Scotland has encouraged submission of red squirrel carcases found by members of the public and various organisations to the Royal (Dick) School of Veterinary Studies at the University of Edinburgh for postmortem examination. This is the first paper to use information obtained via this scheme. The aim of this study was to provide detailed information on the causes of death in red squirrels in Scotland, and the demographic variables associated with these squirrels, in order to highlight factors that might be threats to Scottish red squirrel populations.

Materials and methods

Study population and pathological examination

Two hundred and sixty-six dead red squirrels, found in Scotland between September 29, 2005 and July 24, 2009, were submitted to the Veterinary Pathology Unit at the University of Edinburgh by members of the public, red squirrel conservation organisations, ranger services and veterinary surgeries, for postmortem examination. Four of these carcases were omitted from the data set because their condition was too poor to allow meaningful assessment, leaving 262 squirrels.

The administrative region and Ordnance Survey grid reference where each squirrel was found were recorded, if known. The specific area in which each squirrel was found was recorded, if known, as in a garden, in a woodland, on/near a road, at the base of a tree, on/near a squirrel feeder, or in some other area; and it was also recorded, if known, whether the squirrel had been found dead, had died after having been found ill or injured, or was euthanased after having been found ill or injured. For some of the squirrel carcases there was incomplete or no information regarding the date of finding, location and/or situation in which they were found; squirrel carcases with no specific location information were assumed to have come from the area around the sender's address.

The carcases were frozen at –20°C on arrival if the postmortem examination was not scheduled to be carried out immediately. The postmortem condition (fresh, moderately decomposed or decomposed) at the time of the examination was assessed visually. Decomposition limited the completeness of the postmortem examination in some cases, and therefore the number of animals differs for some of the parameters assessed.

Assessment of the age of the squirrels was based on a previously reported method (Carroll and others 2009). Measurements were made of bodyweight and crown-rump length (the rostral margin of the pinna to the base of the tail). In females, the uterine horn length was measured, and pregnancy status and the degree of mammary development were assessed. In males, the presence or absence of scrotal pigment (which is prominent in mature males), position of the testes (scrotal or abdominal, with scrotal testes consistent with maturity) and length and width of the testes were recorded. Each squirrel was classified as adult, subadult or juvenile on the basis of its bodyweight, crown-rump length and the degree of maturity of the hair coat and reproductive organs. Body condition was determined by palpation of the hindlimb and lumbar musculature, and the squirrels were categorised as fat, normal, thin or emaciated. The presence of any fleas, ticks or lice was noted, along with the degree of infestation (mild, moderate or severe).

A full postmortem examination was undertaken where the condition of the carcase permitted this, and all macroscopic lesions were recorded. In most squirrels, a PCR for SQPV was carried out on the eyelid, forefoot digital skin and lip skin. In cases where blood or body cavity fluid was obtainable, an ELISA for SQPV antibody was also performed, as described by McInnes and others (2009). When SQPV infection was suspected, confirmation was obtained by electron microscopy of scabs to demonstrate the characteristic virus particles (McInnes and others 2009). In two cases, histopathological examination was undertaken of haematoxylin and eosin-stained sections of buffered formalin-fixed, paraffin wax-embedded tissues, using standard methods. The postmortem findings, history, results of the virological examination described and, in the two cases specified, histopathological examination, were used to determine, where possible, the predominant cause of death (or cause of signs that led to euthanasia) for each of the 262 squirrels.

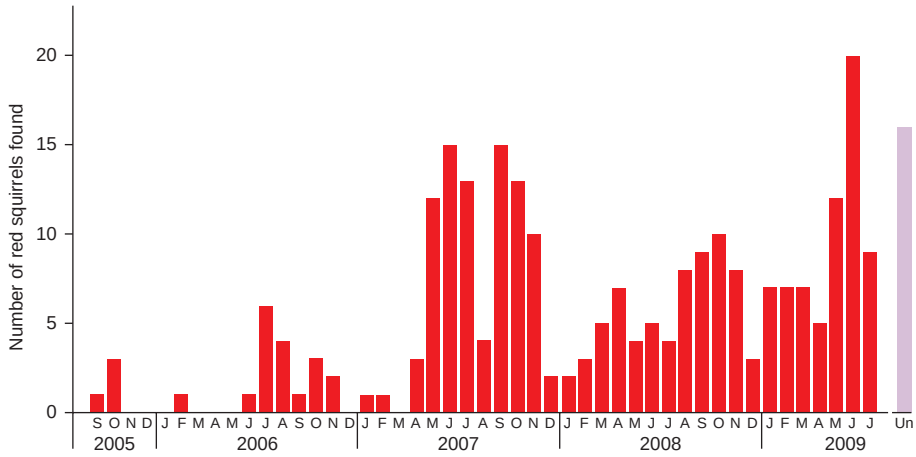


FIG 2: Numbers of Scottish red squirrel carcases (n=262) submitted for postmortem examination between September 2005 and July 2009, categorised by the date on which they were found. Un Month of submission was unknown

Data analysis

Minitab 15 was used for statistical analysis of the data. Associations between categorical variables were examined using chi-squared analysis where appropriate. Fisher's exact test was performed if expected counts were less than five. In all cases $P<0.05$ was taken to indicate statistical significance.

Results

Sample population

The dead red squirrels submitted for examination were from various regions

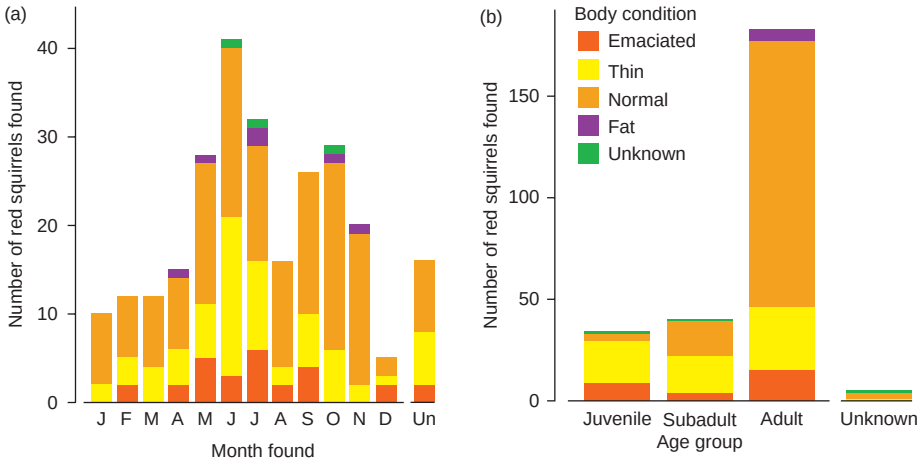


FIG 3: Body condition of 262 red squirrel carcases from Scotland (a) in terms of percentage by month, and (b) within each of three age groups, reported between September 2005 and July 2009. Un Month of submission unknown

throughout Scotland; however, a majority (175 of 262; 66.8 per cent) came from Dumfries and Galloway. The precise locations, based upon grid reference numbers, were known for 247 of the squirrels (Fig 1); the majority of the squirrels were found associated with the road network (data not shown).

The month of discovery was known for 246 of the 262 squirrels. The numbers of squirrels submitted for examination increased overall throughout the study period (Fig 2).

TABLE 1: Numbers of red squirrel carcases found in Scotland, categorised by age group and sex, which were submitted for postmortem examination between September 2005 and July 2009

	Juvenile	Subadult	Adult	Age group unknown	Total
Female	14	17	91	1	123
Male	20	21	92	3	136
Sex unknown	0	2	0	1	3
Total	34	40	183	5	262

Information with regard to the exact location of the animal was available for 154 of the 262 squirrels. Of these, 41 per cent were known to have been found on or near a road, 18 per cent in a garden, 12 per cent within a wooded area, 9 per cent at the base of a tree and 4 per cent in or near a feeder; 16 per cent had been found in a variety of other locations. Information on whether the squirrel was found dead, died after being found ill or injured, or was euthanased after being found ill or injured was available for 226 of the 262 squirrels. Seventy per cent of these 226 squirrels had been found dead, 15 per cent had died after being found ill or injured, and 15 per cent had been euthanased as a result of illness or injury.

The age groups of 257 of the squirrels and the sex of 259 of them could be determined. The majority of the squirrels were adult (71 per cent) and 48 per cent were female (Table 1); six of the females were pregnant. Among the carcases examined, there was no statistically significant association between age group and sex (P=0.603). Body condition was determined in 259 of the 262 squirrels. Of these, most were in normal body condition (60 per cent), and 38 per cent were either thin or emaciated; emaciated animals were mainly found from May to October (Fig 3a). There was a statistically significant association between age group and body condition (P<0.001) (Fig 3b), with a higher proportion of juvenile and subadult squirrels being thin or emaciated (88 per cent), and a much lower proportion of adults in these categories (25 per cent).

Assigned causes of death

On the basis of the gross postmortem findings, and in two cases histopathological examination, it was possible to assign a likely predominant cause of death in 245 (94 per cent) of the 262 squirrels. Of the remaining 17 carcases, two were in too poor a condition for a predominant cause of death to be determined, and 15 appeared healthy with no significant abnormalities found at postmortem examination. The assigned causes of death are shown in Table 2 and Fig 4a; road traffic

TABLE 2: Summary of the causes of death of 245 red squirrels for which a predominant cause of death could be assigned

Assigned cause of death	Description	Number (%) of squirrels
Road traffic accident	Clear evidence of trauma and found on or near a road	105 (42.9)
Squirrelpox virus infection	Typical skin ulceration and scab formation, confirmed by electron microscopy	35 (14.3)
Trauma	Clear evidence of trauma but not found near a road	27 (11.0)
Starvation	Emaciated body condition and no other predominant cause of death	24 (9.8)
Pneumonia	Consolidation of lung lobes with or without pleuritis	18 (7.3)
Enteropathy		12 (4.9)
Intussusception	Colonic or colorectal intussusception	5 (2.0)
Enteritis	Mucosal congestion and/or accumulation of fluid ingesta or gas in the intestine. In some cases, diarrhoea present	4 (1.6)
Small intestinal foreign body	Vegetable foreign body penetrating the intestinal wall	1 (0.4)
Unspecified	Accumulation of fluid ingesta or gas in the intestine without congestion or diarrhoea	2 (0.8)
Abscess	Digit (1); kidney (1); submandibular (1); intrathoracic (2)	5 (2.0)
Ectoparasitism	Severe louse infestation with grossly evident anaemia	4 (1.6)
Other infections		4 (1.6)
Balantitis	Prepuce severely inflamed with purulent discharge	1 (0.4)
Pyometra	Pus accumulation in distended uterus	1 (0.4)
Severe conjunctivitis	Conjunctivitis with keratitis in one eye and bilateral purulent ocular discharge	1 (0.4)
Septicaemia	Enlarged lymph nodes and spleen, epicardial haemorrhages	1 (0.4)
Died/euthanased for other reasons		11 (4.5)
Neoplasia	Suspected soft tissue sarcoma with pulmonary metastases; multicentric lymphoma	2 (0.8)
Electrocution	Found under powerline with subcutaneous haemorrhages/oedema and internal haemorrhage	2 (0.8)
Cardiac failure	Cardiomegaly with left ventricular hypertrophy; cardiomegaly with atrial dilation	2 (0.8)
Suspected fatal allergic reaction	Severe subcutaneous oedema of pinnae, muzzle, eyelids, limbs, scrotum and tail	1 (0.4)
Stress	No gross abnormalities but squirrel recently caught for translocation	1 (0.4)
Urinary obstruction	Severe bladder distension and apparent urethral obstruction	1 (0.4)
Congenital hindlimb contracture	Partially contracted hindlimbs with abnormal angulation	1 (0.4)
Suspected anticoagulant rodenticide poisoning	Multiple internal and external haemorrhages, history of possible access to rodenticide	1 (0.4)

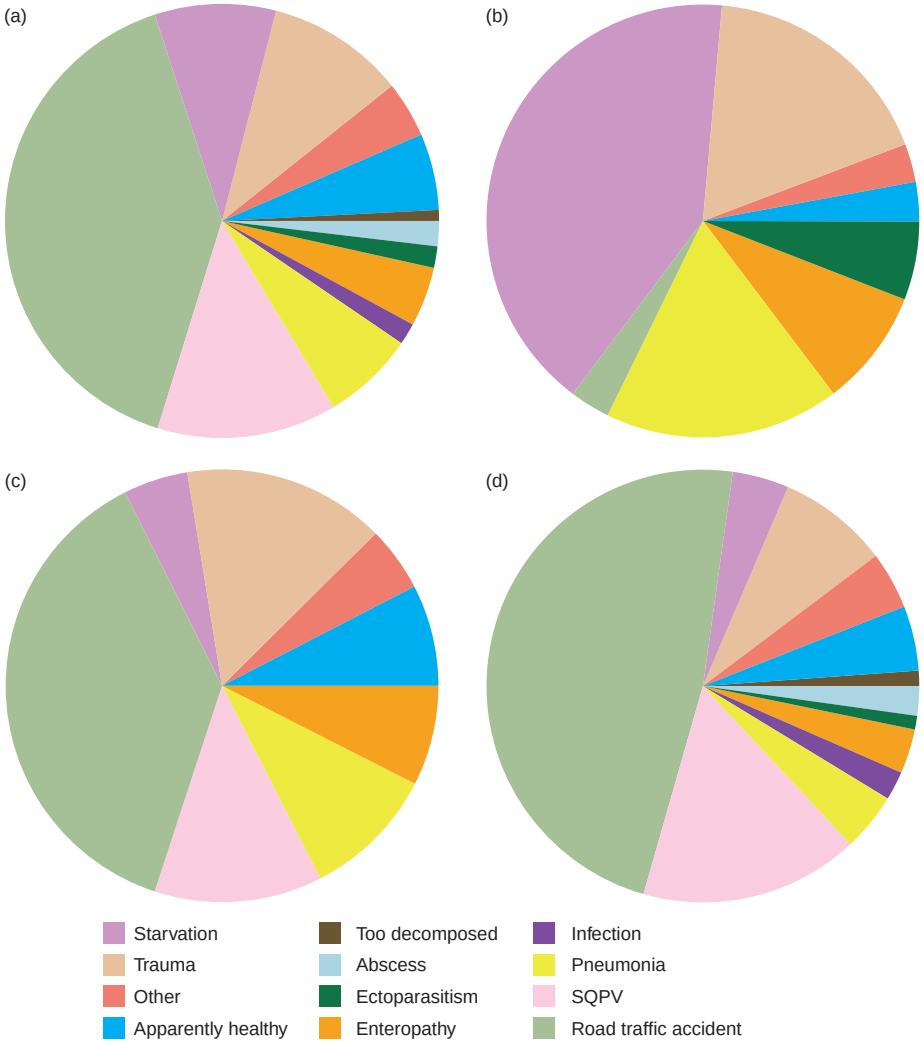


FIG 4: Predominant assigned causes of death in red squirrel carcases found in Scotland between September 2005 and July 2009 and submitted for postmortem examination. (a) All squirrels in the study (n=262), (b) juveniles (n=34), (c) subadults (n=40), (d) adults (n=183)

accidents, squirrelpox, trauma and starvation together represented 78 per cent of the assigned causes of death.

There was a statistically significant association between age group and predominant cause of death ($P<0.001$). Road traffic accidents were rare in the juvenile group (Fig 4b), and no juveniles had died of squirrelpox. Starvation was the most common cause of death in juveniles, followed by pneumonia and trauma (Fig 4b). This difference was less marked between subadults (Fig 4c) and adults (Fig 4d), with road traffic accidents being the most common in both age groups, followed by trauma, squirrelpox and pneumonia in the subadults, and squirrelpox and trauma in the adults (Fig 4d). There was a statistically significant association between body condition and predominant cause of death ($P<0.001$); 77 per cent of thin or emaciated squirrels died from pneumonia, squirrelpox or starvation, compared with 17 per cent of normal/fat squirrels, whereas 83 per cent of normal/fat squirrels died from road traffic accidents or trauma.

The number of submissions per region (Fig 1) was a confounding factor, and the predominant cause of death varied according to the location in which they had been found (Fig 5). No SQPV-associated disease was reported outside Dumfries and Galloway, and the majority of submissions from central Scotland related to road traffic accidents. Road traffic accidents were also the largest grouping in the animals submitted from the Borders and Dumfries and Galloway.

External parasites

Among the 262 squirrels examined, external parasites (fleas, ticks and/or lice) were observed on 29 per cent. Fifty-six per cent of the squirrels with external parasites were found to have fleas alone, 12 per cent had ticks alone, and 12 per cent had lice alone; 15 per cent had both fleas and ticks, 4 per cent had both ticks and lice, and 1 per cent had both fleas and lice. There was a statistically significant association between body condition and the presence of external parasites ($P=0.027$), with 38 per cent of thin or emaciated squirrels having external parasites, compared with 25 per cent of normal/fat squirrels. In only four cases was ectoparasitism considered the likely cause of death; all four were juveniles with many hundreds of lice and severe pallor of the carcase, consistent with anaemia.

SQPV

No squirrelpox cases were seen from the start of the study period (September 2005) until May 2007, when two cases were submitted. In July 2007 there was one case, in March 2008 and June 2008 there were two cases each, and in August 2008 there was one case. In 2009 there was at least one case every month, with one case each month from January through to April, then seven cases in May, 13 cases in June, and three cases in July. Of the 35 squirrels confirmed positive for SQPV infection, 33 had shown obvious clinical or postmortem signs of the disease.

There was no statistically significant association between SQPV infection and sex ($P=0.340$). However, there was a statistically significant association between the presence of SQPV infection and age group ($P=0.015$), with 86 per cent of confirmed squirrelpox cases being adults and no cases in juveniles. In addition, there was also a statistically significant association between SQPV infection and body condition ($P=0.011$), with 57 per cent of SQPV-infected squirrels being thin or emaciated. There was a statistically significant association between SQPV infection and the presence of external parasites ($P=0.007$), with 49 per cent of SQPV-infected squirrels carrying external parasites, compared with only 26 per cent of uninfected squirrels. Specifically, there was a statistically significant association between SQPV infection and the presence of fleas ($P=0.001$), with 43 per cent of SQPV-infected squirrels carrying fleas, compared with 18 per cent of the uninfected squirrels; however, there was no significant association between SQPV infection and the presence of ticks or lice ($P>0.226$).

Discussion

Convenience sampling was the only practical way to obtain the squirrels examined in this study. As a consequence, the results are unlikely to be representative of the whole Scottish red squirrel population. Bias was undoubtedly introduced in the way in which the squirrels were collected, because squirrels that had died within areas visible to the public were more likely to be discovered. Similarly, more squirrels are likely to be submitted during times of the year when members of the public are more active outdoors. Therefore the numbers of squirrels submitted by month do not necessarily reflect actual fluctuations in mortality. In addition, the majority of the squirrel carcases were submitted from Dumfries and Galloway. This is most likely because of the greater concern about SQPV infection among the public and countryside rangers in this region. Nevertheless, this study is the first to document a (non-exhaustive) list of likely causes of death from a large sample size of red squirrels in Scotland, and to indicate how some of these causes relate to the

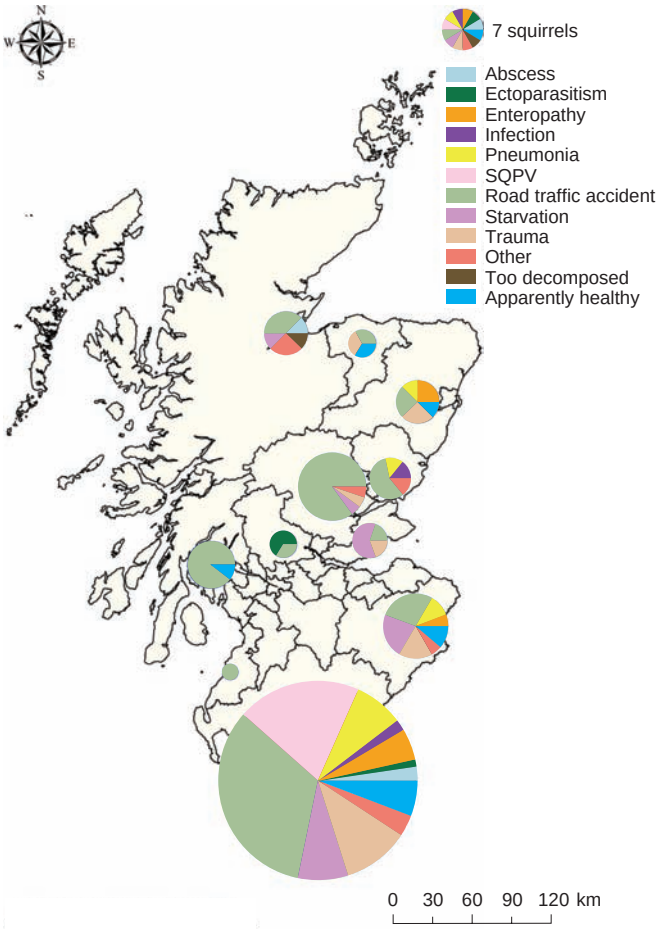


FIG 5: Cause of death, assigned from findings at postmortem examination of 258 red squirrels found between September 2005 and July 2009, categorised according to the county from which the submissions came. The size of each pie chart represents the number of squirrels submitted from that county, and the location of the county is the average location for squirrels submitted from that county. Crown Copyright/database right 2009. An Ordnance Survey/EDINA supplied service

age, body condition and sex of the animals, and the presence of external parasites.

Road traffic accidents were the most common assigned cause of death in the Scottish red squirrels examined in this study, followed by SQPV-associated disease, trauma other than by road traffic accident and starvation. The predominance of road traffic accidents as an apparent cause of death remained when adults and subadults were considered separately, but not in juveniles, in which starvation was the predominant cause of death. Adults and subadults range more widely, and this may result in them crossing roads, whereas juveniles are more likely to be found near the nest area. Efforts to decrease the number of red squirrel road casualties have been incorporated into conservation efforts in some areas; for example, aerial squirrel crossings have been built over roads on the island of Jersey; however, the true impact of these crossings on the squirrel population is not known (Gurnell and Magris 2002). The majority of the juveniles that had apparently died of starvation were found during the late summer or early autumn, corresponding to the season in which summer-born young are weaned (Lurz and others 2005). This newly weaned status was frequently corroborated by postmortem examination, and presumably reflects failed adaption to independent life. Starvation of this type does not apply to subadults or adults because they have long since been weaned, and therefore an apparent inability to use food sources, rather than any actual shortage of food sources, could explain why starvation was one of the more significant causes of death. Provision of supplementary food has been shown in some cases to increase the population density of squirrels in a given area (Gurnell and Magris 2002); however, increased population density also has the potential to support a higher level of disease and spread of parasites. Since the spread of SQPV in particular is known to be a threat to red squirrel populations (Tompkins and others 2002, Rushton and others 2006), any attempt to increase squirrel population density through food supplementation may be detrimental rather than helpful in areas where there is a risk of SQPV being present.

A higher than expected proportion of thin or emaciated squirrels was found to have died, apparently from pneumonia, squirrelpox or starvation. By definition, any squirrel that had died of starvation would have been in poor body condition at the time of death. Squirrels that died of debilitating disease conditions, such as squirrelpox or severe ectoparasitism, would reasonably be expected to have lost body condition before death. The possibility also exists that squirrels in thin or emaciated condition are more susceptible to disease. Demas and others (2003) found that a decrease in total body fat can reduce humoral immunity in Siberian hamsters (*Phodopus sungorus*) and prairie voles (*Microtus ochrogaster*); a later study of Siberian hamsters (Demas and Sakaria 2005) found this change in immunity to be regulated through levels of leptin, an adipose tissue hormone. In the present study, it was not known whether the squirrels that died of disease were in poor body condition before or after succumbing to disease, but poor body condition is likely to have been a predisposing factor to disease in at least some of the squirrels.

There was also an association between body condition and the presence of external parasites, with more thin or emaciated animals having external parasites compared with animals in normal or fat body condition. It is not known what proportion of these squirrels were in poor body condition before becoming infested with external parasites, and what proportion lost body condition as a result of already established ectoparasitism. Depression of immunity as a result of poor body condition may have contributed to the establishment of ectoparasitism in some of these squirrels; however, the infestation was severe enough to be the suspected cause of death only in four of the animals.

The presence of SQPV disease was first detected during the present study in May 2007; there had been no previous reported cases of SQPV in red squirrels in Scotland before that date (McInnes and others 2009). It is notable that, since the study period described by McInnes and others (2009), SQPV has increased in importance as a cause of mortality among red squirrels in Scotland. Nearly all of the squirrels that were confirmed as infected with SQPV had clearly visible pox lesions at postmortem examination, although in two cases SQPV infection was not suspected on postmortem examination. These two cases were considered to be early cases, and highlight the fact that infection may be overlooked in the absence of laboratory investigation. There was a significant association between SQPV infection and thin or emaciated body condition. It has been shown that affected red squirrels can lose weight as a result of SQPV disease (Tompkins and others 2002). However, it is not yet known whether squirrels in poor body condition when they come into contact with the virus are more susceptible to squirrelpox disease than squirrels in normal body condition.

It is still uncertain whether SQPV can be carried by vectors such as fleas, ticks or lice; Sainsbury and others (2008) concluded that epidemiological patterns of SQPV infection in red squirrels did not indicate that the disease was vectorborne. In the present study, SQPV-infected squirrels were more likely to have external parasites, specifically fleas. However, the converse was not true; that is, squirrels with parasitic infestation were not more likely to be infected with SQPV. This could suggest that SQPV infection might predispose squirrels to external parasitism, rather than that the presence of external parasites predisposes to SQPV infection. However, the association between fleas and squirrelpox must be treated with caution because it is possible that more squirrels with squirrelpox were euthanased by veterinary surgeons and immediately placed in a bag, preventing loss of fleas from the carcases.

Finally, there was no statistically significant association between sex and SQPV infection. This finding is contrary to findings of a previous study which examined past squirrelpox epidemics outside Scotland, and concluded that males may be more likely than females to be infected (Sainsbury and others 2008).

In conclusion, this study brings to light many different causes of mortality in red squirrels in Scotland, and the demographic factors associated with these. It demonstrates the value of schemes that gather information about causes of red squirrel mortality, some of which may not be as obvious as SQPV disease. Information gathered from wildlife submission schemes and studies such as this have the potential to aid in the detection of new disease patterns or other threats to populations, and may ultimately help to direct conservation efforts.

Acknowledgements

The authors are grateful to all of the red squirrel conservation groups, ranger services, veterinary surgeons and members of the public who submitted squirrel carcases, including Richard Wales, Stephanie Johnstone and Ann-Marie McMaster of Red Squirrels in South Scotland, the Buccleuch Ranger Service, Tayside Red Squirrel Forum, Nithsdale Veterinary Surgeons, the Bard Veterinary Group and Elly Hamilton. The authors are also grateful to the Wellcome Trust for a Wellcome Vets vacation scholarship (J. LaRose) and to Scottish Natural Heritage for providing part of the funding for this project.

References

CARROLL, B., RUSSELL, P., GURNELL, J., NETTLETON, P. & SAINSBURY, A. W. (2009) Epidemics of squirrelpox virus disease in red squirrels (*Sciurus vulgaris*): temporal and serological findings. *Epidemiology and Infection* **137,** 257-265

DEMAS, G. E., DRAZEN, D. L. & NELSON, R. J. (2003) Reductions in total body fat decrease humoral immunity. *Proceedings of the Royal Society B. Biological Sciences* **270,** 905-911

DEMAS, G. E. & SAKARIA, S. (2005) Leptin regulates energetic tradeoffs between body fat and humoral immunity. *Proceedings of the Royal Society B. Biological Sciences* **272,** 1845-1850

DUFF, J. P., HIGGINS, R. & FARRELLY, S. (2007) Enteric adenovirus infection in a red squirrel (*Sciurus vulgaris*). *Veterinary Record* **160,** 384

DUFF, J. P., SCOTT, A. & KEYMER, I. F. (1996) Parapox virus infection of the grey squirrel. *Veterinary Record* **138,** 527

GURNELL, J. & MAGRIS, L. (2002) Population ecology of the red squirrel (*Sciurus vulgaris*) in a fragmented woodland ecosystem on the Island of Jersey, Channel Islands. *Journal of Zoology* **256,** 99-112

HARRIS, S., MORRIS, P., WRAY, S. & YALDEN, D. (1995) A Review of British Mammals: Population Estimates and Conservation Status of British Mammals Other than Cetaceans. Joint Nature Conservation Committee

KENWARD, R. E. & HODDER, K. H. (1998) Red squirrels (*Sciurus vulgaris*) released in conifer woodland: the effects of source habitat, predation and interactions with grey squirrels (*Sciurus carolinensis*). *Journal of Zoology* **244,** 23-32

KENWARD, R. E., HODDER, K. H., ROSE, R. J. & WALLS, C. A. (1998) Comparative demography of red squirrels (*Sciurus vulgaris*) and grey squirrels (*Sciurus carolinensis*) in deciduous and coniferous woodland. *Journal of Zoology* **244,** 7-21

KENWARD, R. E. & HOLM, J. L. (1993) On the replacement of the red squirrel in Britain: a phytotoxic explanation. *Proceedings of the Royal Society B. Biological Sciences* **251,** 187-194

KEYMER, I. F. (1983) Diseases of squirrels in Britain. *Mammal Review* **13,** 155-158

LURZ, P. W. W., GURNELL, J. & MAGRIS, L. (2005) *Sciurus vulgaris*. *Mammalian Species* **769,** 1-10

MCINNES, C. J., COULTER, L., DAGLEISH, M. P., FIEGNA, C., GILRAY, J., WILLOUGHBY, K., COLE, M., MILNE, E., MEREDITH, A., EVEREST, D. J. & MACMASTER, A. M. (2009) First cases of squirrelpox in red squirrels (*Sciurus vulgaris*) in Scotland. *Veterinary Record* **164,** 528-531

MCINNES, C. J., WOOD, A. R., THOMAS, K., SAINSBURY, A. W., GURNELL, J., DEIN, F. J. & NETTLETON, P. F. (2006) Genomic characterization of a novel poxvirus contributing to the decline of the red squirrel (*Sciurus vulgaris*) in the UK. *Journal of General Virology* **87,** 2115-2125

RUSHTON, S. P., LURZ, P. W., GURNELL, J., NETTLETON, P., BRUEMMER, C., SHIRLEY, M. D. & SAINSBURY, A. W. (2006) Disease threats posed by alien species: the role of a poxvirus in the decline of the native red squirrel in Britain. *Epidemiology and Infection* **134,** 521-533

SAINSBURY, A. W., DEAVILLE, R., LAWSON, B., COOLEY, W. A., FARELLY, S. S., STACK, M. J. & OTHERS (2008) Poxviral disease in red squirrels *Sciurus vulgaris* in the UK: spatial and temporal trends of an emerging threat. *Ecohealth* **5,** 305-316

SIMPSON, V. R., BIRTLES, R. J., BOWN, K. J., PANCIERA, R. J., BUTLER, H. & DAVISON, N. (2006) *Hepatozoon* species infection in wild red squirrels (*Sciurus vulgaris*) on the Isle of Wight. *Veterinary Record* **159,** 202-205

SIMPSON, V. R., DAVISON, N. J., BORMAN, A. M., LINTON, C. J. & EVEREST, D. (2009) Fatal candidiasis in a wild red squirrel (*Sciurus vulgaris*). *Veterinary Record* **164,** 342-344

TOMPKINS, D. M., SAINSBURY, A. W., NETTLETON, P., BUXTON, D. & GURNELL, J. (2002) Parapoxvirus causes a deleterious disease in red squirrels associated with UK population declines. *Proceedings of the Royal Society B. Biological Sciences* **269,** 529-533

USHER, M. B., CRAWFORD, T. J. & BANWELL, J. L. (1992) An American invasion of Great Britain: the case of the native and alien squirrel (*Sciurus*) species. *Conservation Biology* **6,** 108-115

PAPERS

First cases of squirrelpox in red squirrels (*Sciurus vulgaris*) in Scotland

C. J. McInnes, L. Coulter, M. P. Dagleish, C. Fiegna, J. Gilray, K. Willoughby, M. Cole, E. Milne, A. Meredith, D. J. Everest, A-M. MacMaster

Squirrelpox, caused by a poxvirus, is a major threat to the remaining UK red squirrel population. The spread of antibody-positive grey squirrels has been monitored in the UK for the past decade. In 2005 grey squirrels that had been exposed to the virus appeared in the south of Scotland for the first time, followed approximately two years later by the appearance of squirrelpox disease in the local red squirrels. Four squirrels were examined. They all had gross external lesions and histological lesions typical of squirrelpox disease, but no significant internal lesions. The diagnosis was confirmed by PCR, electron microscopy and serology.

THE red squirrel (*Sciurus vulgaris*) has been in serious decline in the UK for the last 90 years with only a few isolated populations remaining in England and Wales. It is estimated that there are approximately 161,000 red squirrels left and that 75 per cent of them are in Scotland, but there too the population is decreasing (Harris and others 1995). As the red squirrel population has been in decline, the grey squirrel (*Sciurus carolinensis*) population has expanded rapidly, after the introduction of a small number of animals from the USA to various parts of the UK in the late 19th and early 20th centuries (Middleton 1930, Shorten 1954, Lloyd 1983, Rushton and others 2006). Anecdotal evidence suggested that the local disappearance of red squirrels often coincided with the emergence of grey squirrels in the vicinity, but it was not until the 1930s that it was formally recognised that grey squirrels could be having a direct effect on the red squirrel population (Middleton 1930).

Several reports have linked the local extinction of red squirrels to disease epidemics (Middleton 1930, Shorten 1954, Edwards 1962, Vizoso 1968, Keymer 1983). Some of the diseases were described as myxomatosis-like and others were described variously as 'mange', 'distemper' and 'consumption'. The only pathogens isolated from diseased red squirrels were *Eimeria* species and a parainfluenza virus, neither of which was thought to be responsible for the myxomatosis-like disease. Some authors linked the epidemics to the appearance of grey squirrels in the same woodland, whereas others suggested that grey squirrels were not known to be present anywhere in the vicinity. Middleton (1930) suggested that grey squirrels might be the carriers of a lethal disease that affected red squirrels to which they themselves were immune. Scott and others (1981) isolated a poxvirus from a dead red squirrel and linked it to the extinction of the red squirrel population in Norfolk, but there were doubts that the poxvirus was connected with the decline of red squirrels in other parts of the UK (Reynolds 1985). However by the late 1990s, after further outbreaks of poxvirus-associated disease (Sainsbury and Gurnell 1995, Sainsbury and Ward 1996) and by using an ELISA to detect specific antibodies against the virus, it was recognised that red squirrels almost certainly died if they contracted the poxvirus whereas grey squirrels appeared to be unaffected (Sainsbury and others 2000). Tompkins and others (2002) demonstrated that experimental infections did not result in pathological changes in grey squirrels, but that red squirrels rapidly became ill, losing their appetite and condition to such an extent that it was assumed that they would not survive an infection with the virus in the wild.

At first, the virus was classified as a parapoxvirus (Scott and others 1981) owing to the pathological changes being confined to the skin and the morphological appearance of the virus when visualised by electron microscopy, but an investigation of the viral genome revealed that although it was undoubtedly a poxvirus, the virus did not belong within any of the recognised genera of the poxvirus family. It is now known as squirrelpox virus (SQPV) and should be reclassified, on its own, into a separate genus of the poxvirus family (Thomas and others 2003, McInnes and others 2006).

A serological study of grey squirrels from across the UK found that approximately 60 per cent in England and Wales had been exposed to SQPV, but that the grey squirrels in Scotland were seronegative (Sainsbury and others 2000). This is consistent with the fact that no SQPV-associated disease had been reported in Scotland. In fact, cases of disease in red squirrels appear to be confined to areas of the UK where there are seropositive grey squirrels, providing further circumstantial evidence that grey squirrels may act as a reservoir of SQPV and transmit it to the red squirrels (Rushton and others 2000).

This paper reports the appearance of SQPV seropositive grey squirrels in the south of Scotland followed by cases of fatal disease in red squirrels two years later.

Veterinary Record (2009) **164,** 528-531

C. J. McInnes, BSc, PhD,
L. Coulter, BSc, PhD,
M. P. Dagleish, BVM&S, PhD, MRCVS,
C. Fiegna, DVM,
J. Gilray,
K. Willoughby, BVMS, PhD, MRCVS,
Moredun Research Institute, Pentlands Science Park, Bush Loan, Edinburgh EH26 0PZ
M. Cole, BSc, PhD,
Scottish Natural Heritage, Silvan House, 231 Corstorphine Road, Edinburgh EH12 7AT.

E. Milne, BVM&S, PhD, DipECVCP, DipRCPath, FRCVS,
A. Meredith, MA, VetMB, CertLAS, CertZooMed, MRCVS,
Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush Veterinary Centre, Roslin, Midlothian EH25 9RG
D. J. Everest,
Veterinary Laboratories Agency – Weybridge, New Haw, Addlestone, Surrey KT15 3NB
A-M. MacMaster, BSc,
Red Squirrels in South Scotland, Carlow House, Locharbriggs, Dumfries DG1 1QS

E-mail for correspondence: colin.mcinnes@moredun.ac.uk

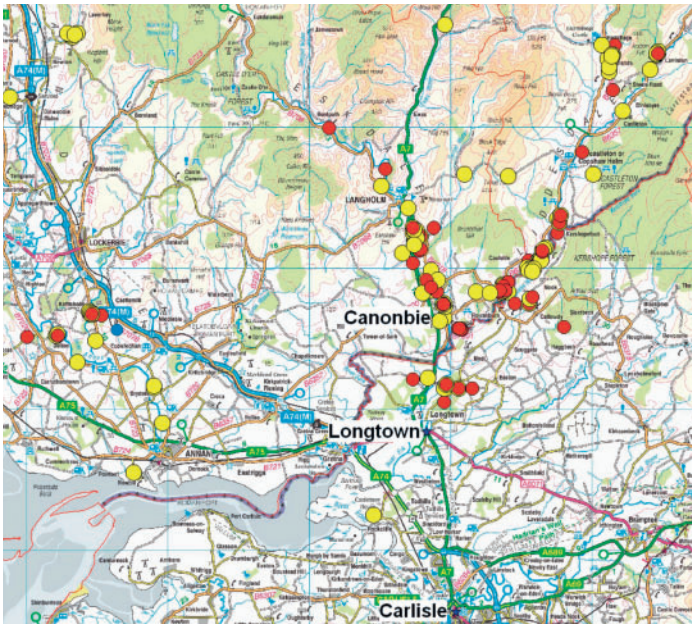


FIG 1: Sites where squirrelpox virus seropositive grey squirrels (*Sciurus carolinensis*) (red circles) and seronegative grey squirrels (yellow circles) were detected before May 2007; the site where the index red squirrel (*Sciurus vulgaris*) case was found is indicated by the blue circle

FIG 2: First red squirrel (*Sciurus vulgaris*) (R597) to be found in Scotland suffering from squirrelpox; the erythematous scabs on the periorbital skin and feet are typical of squirrelpox lesions. The squirrel was found alive, but in a stressed condition and was euthanased by a veterinary practitioner

Materials and methods

Squirrelpox virus ELISA

Blood samples were collected from grey squirrels culled in the north of England and south of Scotland by local landowners, government agencies and red squirrel conservation bodies. The samples were centrifuged at 2000 *g* for five minutes and the serum was examined for the presence of antibodies against SQPV as described by Sainsbury and others (2000). Briefly, SQPV antigen was coated on to 96-well plates and incubated with serum samples collected from grey and red squirrels from southern Scotland. Non-specifically bound protein was then removed by washing and anti-SQPV IgG was detected by incubating with horseradish peroxidase (HRP)-conjugated protein G and developing colour with 3,3′, 5,5′-tetramethylbenzidine peroxidase substrate (SureBlue; Kirkegaard and Perry Laboratories); the reaction was stopped after five minutes by the addition of 0·18M sulphuric acid and read at an optical density of 450 nm with a Dynex MRX platereader. An optical density of 0·2 above the sample background was used as the cut-off to discriminate between positive and negative results. Ordinance survey grid references were available for each of the blood samples so that the location of the squirrels could be marked on a map.

Red squirrels

On May 8, 2007 a sick red squirrel was found near Lockerbie. It was lethargic, had difficulty in moving around and had skin lesions suggestive of squirrelpox. The animal was euthanased and examined postmortem. During the next week three more red squirrels showing clinical signs suggestive of SQPV infection were found dead in the same general locality. They were also examined postmortem.

The three males and one female were weighed and tissue samples were taken, including multiple skin samples. The internal organs and half the skin samples were fixed in buffered formalin for histological examination and the other skin samples were frozen until required for the detection of SQPV by PCR. The samples for histological examination were embedded in paraffin wax and 5 µm sections were mounted on glass microscope slides and stained with haematoxylin and eosin.

Electron microscopy

Electron microscopy was used to examine tissue from the periorbital area. The tissue was ground in approximately 2 ml of 0·1M Sorenson's phosphate buffer (pH 6·6) and 50 µl samples of the tissue suspension were dried on to copper/rhodium formvar/carbon-coated support grids, which had been subjected to a plasma glow discharge. The grids were then negatively stained with 2 per cent phosphotungstic acid (pH 6·6) and examined in a Phillips CM10 transmission electron microscope at 80 kV.

PCR analysis

The A11L gene of SQPV (McInnes and others 2006) was detected by PCR using the primers SQ026F 5′-ATGTCAGTCACGATAAGATT-3′ and SQ026R 5′-TCATGTCAGTCGGGTGATGA-3′ that were designed to amplify a 258 bp fragment of the SQPV genome. Poxviral orthologues of this gene are found only in the parapoxivinae and molluscom contagiosum virus. DNA was isolated from the tissue by using the QIAGEN DNeasy Kit according to the manufacturer's instructions. One microlitre of the resulting DNA was incubated with 1µM of each PCR primer, 200µM dNTPs, 1·5mM $MgCl_2$, 10mM Tris-HCl pH 8·3 and 50mM KCl and 1 unit Taq DNA Polymerase (Roche). DNA was denatured by incubating at 94°C for four minutes and amplified using 30 cycles of 94°C for one minute, 55°C for 30 seconds and 72°C for one minute, followed by a final incubation at 72°C for five minutes. DNAs isolated from vaccinia virus, orf virus, pseudocowpox virus, bovine papular stomatitis virus, sealpox virus and from a squirrelpox-negative red squirrel were used as negative controls, and DNA from squirrelpox isolate R1296/99 (Thomas and others 2003) was used as a positive control. The amplification products were electrophoresed in a 1 per cent agarose gel, stained with GelRed (Biotium) and visualised by UV transillumination.

Results

Serology

Before May 2005 approximately 240 grey squirrel sera, collected over the preceding four years from across the Scottish Borders, had been tested for antibodies to the squirrelpox virus and none had been positive. However, between the end of May and the end of June 2005, four grey squirrels from approximately the same locality, south of Newcastleton, were found to be seropositive with respect to SQPV antibodies, the first time SQPV-antibody-positive grey squirrels had been detected in Scotland. During the next two years more seropositive grey squirrels were found spreading north along the river corridors of the Esk and the Annan and the Liddel Water, becoming established in the Scottish Borders and reaching as far as The Hermitage in the east, Langholm to the north and Lockerbie to the north west (Fig 1). By the end of April 2007 43 SQPV seropositive grey squirrels had been detected, but, despite a heightened awareness in both the general public and in conservation organisations of the possible consequences, no SQPV-associated disease was reported in the resident red squirrel population, although the disease had been recognised a few miles south, in Cumbria, for several years (Rushton and others

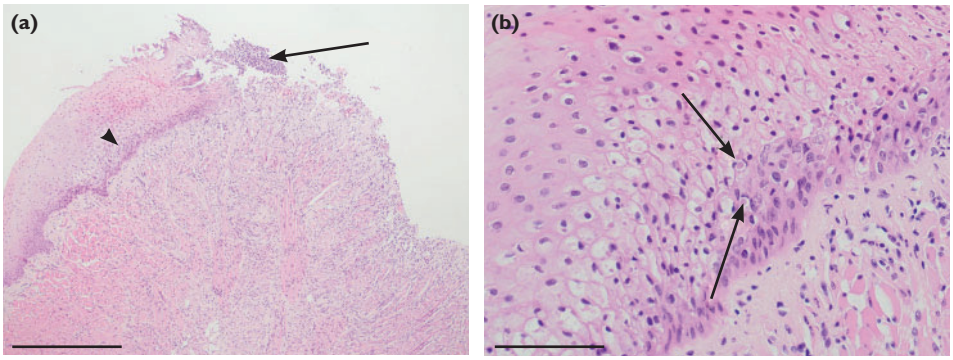


FIG 3: (a) Section of the tongue of red squirrel (*Sciurus vulgaris*) R597 (dorsal surface at top) showing full thickness ulceration of the epidermis (arrow) and an area of ballooning degeneration near the edge of the lesion (arrowhead). Bar=500 µm. (b) Higher magnification of the area of ballooning degeneration; the lesions are more prominent in the deeper part of the stratum spinosum adjacent to the stratum basale, and there appear to be intracytoplasmic amphophilic inclusion bodies, typical of viral infections such as squirrel pox, in some of the affected cells (arrows). Haematoxylin and eosin. Bar=50 µm

2006). However, in May 2007, disease was detected in red squirrels on an estate near Lockerbie (Fig 2).

Pathological findings in red squirrels

On all the four squirrels examined, scabs had formed on the skin, which were erythematous and/or exudative, particularly on the head, but also variably on areas of glabrous skin such as the feet and prepuce and occasionally on non-glabrous areas (Table 1). There was excoriation and ulceration at a number of these sites. All four squirrels had periorbital lesions, which appeared as though they would have severely impaired their vision. Three of the four squirrels were in thin body condition, but three had stomachs full of normal ingesta, suggesting that they had recently eaten, despite one having deep ulcers on its tongue and another having ulcers on the skin of its mouth. It appeared that they had not starved to death. There were no significant gross abnormalities suggestive of a viral infection in the internal organs. Samples from three of the squirrels were taken for histological examination (R597, R21/07 and R23/07). The affected areas of skin had a mixed inflammatory cell dermatitis, full thickness ulceration with complete loss of epidermis, and many bacteria were present. Ballooning degeneration of the intact epidermis was evident at the edge of some of the lesions (Fig 3) and contained occasional intracytoplasmic amphophilic (R597) or eosinophilic (R21/07 and R23/07) inclusions, consistent with viral inclusion bodies. Squirrel R597 also had a mild, predominantly non-suppurative, interstitial nephritis in both kidneys and a mild, predominantly suppurative, oesophagitis and localised gastritis, indicated by inflammatory cells in the lamina propria and submucosa. Squirrel R21/07 had moderate pulmonary oedema and suspected myocardial necrosis, and squirrel R23/07 also had a mild interstitial nephritis, a focal macrovesicular fatty change in the liver and splenic reactivity and hyperplasia of the submandibular lymph node. Apart from the splenic reactivity and lymphoid hyperplasia, which were probably associated with the immunological response to the severe skin lesions, the other pathological changes were considered to have been incidental and unrelated to squirrelpox and unlikely to have been of clinical significance.

TABLE 1: Summary of the gross postmortem findings in the first four squirrelpox virus-positive red squirrels (*Sciurus vulgaris*) found in Scotland

			Skin lesions							
Squirrel	Sex	Weight (g)	Periorbital	Nasal	Lips	Tongue	Feet	Prepuce/ scrotum	Other area	Digesta in stomach
R597	M	247	+	+	–	+	+	–	+	+
R21/07	M	244	+	+	–	–	+	+	–	+
R23/07	F	274	+	+	+	–	+	N/A	+	+
R52/07	M	250	+	–	+	–	–	–	+	–

Diagnostic tests

Serum from each of the four squirrels was tested by the SQPV ELISA (Sainsbury and others 2000) and found to be positive for antibodies to SQPV. Skin samples, mainly from the periorbital area, from all four squirrels were analysed by negative staining electron microscopy and found to contain poxvirus particles (Fig 4). DNA was isolated from the skin samples and subjected to PCR; an amplification product of the expected size (approximately 260 bp) was obtained from all four squirrels (Fig 5).

Discussion

This is the first report of squirrelpox in red squirrels in Scotland. Each of the four squirrels had gross lesions typical of the disease described by Edwards (1962) and Sainsbury and Gurnell (1995). The diagnosis was confirmed by the detection of antibodies to the virus in the blood of the squirrels, and both the virus and viral DNA in skin lesions from all four animals. There were histological lesions typical of those reported for poxvirus infections in the three squirrels from which samples were examined.

Grey squirrels from northern England and the Borders region of Scotland have been monitored serologically for the last decade (Sainsbury and others 2000, Rushton and others 2006). Until May 2005 this monitoring had suggested that the grey squirrels in Scotland had not been exposed to SQPV. However, in May 2005 the first SQPV-seropositive grey squirrels were detected around the Newcastleton area (Fig 1). Over the next two years increasing numbers of seropositive grey squirrels were found further north, along the river corridors in south-west Scotland, culminating in SQPV-associated disease in red squirrels in May 2007, approximately two years after the first detection of the virus in Scotland. The association between the increase in the number of grey squirrels sero-positive for SQPV and the appearance of clinical disease in red squirrels supports the suggestion that grey squirrels infected with SQPV may have played an important role in the decline of red squirrel populations. It also mimics the situation in Cumbria (Rushton and others 2006), where red squirrel numbers were observed to decline about two to three years after the appearance of grey squirrels in the vicinity. Furthermore, through comparisons with data from areas where SQPV was absent, it was estimated that where the grey squirrels were seropositive for exposure to SQPV they were able to supplant the endemic red squirrel population up to 25 times more quickly than in parts of the country where the grey squirrels were seronegative for SQPV (Rushton and others 2006).

The survival of the red squirrel in the UK is in doubt. Their numbers have been in decline for the last 90 years since the introduction of the American grey squirrel. There are probably many reasons for this decline, but the results of this study and other relatively recent evidence suggest that SQPV, present apparently asymptomatically in the grey squirrel population (Sainsbury and others 2000), may have a serious detrimental effect on the viability of red squirrels (Tompkins and others 2002, Thomas and others 2003) and may explain the local extinction of some red squirrel populations (Sainsbury and Gurnell 1995, Sainsbury and Ward 1996, Rushton and others 2000, Tompkins and others 2003). It is thought that the virus was introduced to the UK with the grey squirrels in the late 19th century and that the pattern of disappearance of red squirrels from northern England is consistent with pathogen-mediated competition accelerating the process (Rushton and others 2000, Tompkins and others 2003). The emergence of the disease in Scotland poses an immediate threat to the remaining UK red squirrel population, although it is not known how

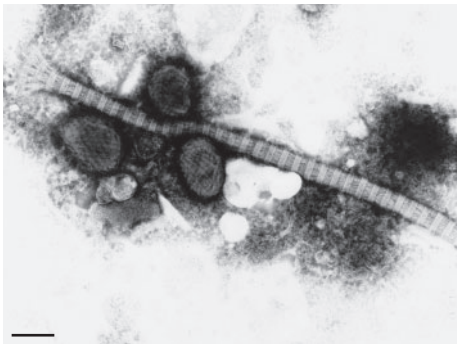


FIG 4: Negative staining electron micrograph of squirrelpox virus particles in the eyelid lesions of red squirrel (*Sciurus vulgaris*) R597, showing several particles lying alongside a collagen fibre. Bar=200 nm

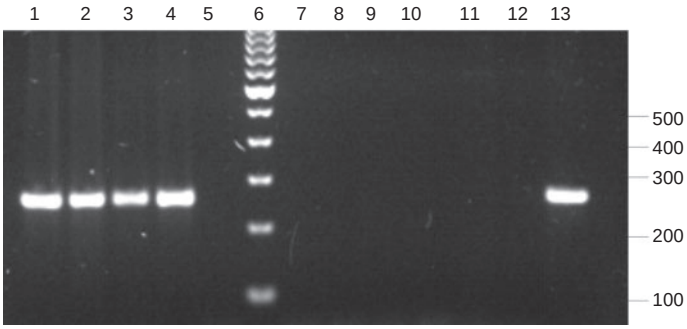


FIG 5: Agarose gel electrophoresis of PCR amplicons obtained from four red squirrels (*Sciurus vulgaris*), using the primers and conditions described in the methods section. The lanes contain DNA from 1 Red squirrel R597, 2 Red squirrel R21/07, 3 Red squirrel R23/07, 4 Red squirrel R52/07, 5 Orf virus, 6 Ready-Load 1 Kb Plus DNA Ladder (Invitrogen), 7 Bovine papular stomatitis virus, 8 Pseudocowpox virus, 9 Vaccinia virus, 10 Sealpox virus, 11 Squirrelpox-negative red squirrel, 12 Water control with no DNA, 13 Red squirrel 1296/99 (Thomas and others 2003). An amplicon estimated to be 260 bp in length is present in lanes 1 to 4 and lane 13

quickly the virus will spread or whether the conservation efforts in the south of Scotland will slow the progress of the virus.

No more cases of disease were reported in the south of Scotland in 2007, but in April 2008 a further outbreak of disease, which is continuing, was reported approximately 20 miles north west of the first outbreak. Between May 2007 and April 2008 six red squirrels have been found with the disease. They may be only a small fraction of those affected because only sick or dead red squirrels in publicly accessible places are likely to have been found and reported. One year after the initial outbreak of squirrelpox there is still a resident population of red squirrels on the Castlemilk Estate, Lockerbie, although no red squirrel sightings have been reported from the 19 ha wood in which three of the diseased red squirrels were found. It is too early to know whether the red squirrels in this area will survive. However, a serological analysis of the current red squirrel population in the area would help to determine whether red squirrels can survive an SQPV infection in the wild and may also help to predict whether there might be an immune population of red squirrels. Until this is known, and the epidemiology of the disease, including any factors influencing the intra- and interspecies transmission of the virus, is better understood, it will be difficult to know how best to protect the red squirrels. The current strategy of trying to create woodland environments that do not disadvantage the red squirrels in the presence of grey squirrels must be continued until more evidence is available on whether vaccination, or other control strategies against the virus, might provide a viable alternative.

Acknowledgements

This work was funded by the Scottish Government and Scottish Natural Heritage. Thanks also go to Hazel Simm for photography and the many conservation groups in northern England and southern Scotland that are actively monitoring the spread of squirrelpox.

References

EDWARDS, F. B. (1962) Red squirrel disease. *Veterinary Record* **74,** 739

HARRIS, S., MORRIS, P., WRAY, S. & YALDEN, D. (1995) A Review of British Mammals: Population Estimates and Conservation Status of British Mammals Other than Cetaceans. JNCC

KEYMER, I. F. (1983) Diseases of squirrels in Britain. *Mammal Review* **13,** 155-158

LLOYD, H. G. (1983) Past and present distribution of red and grey squirrels. *Mammal Review* **13,** 69-80

MCINNES, C. J., WOOD, A. R., THOMAS, K., SAINSBURY, A. W., GURNELL, J., DEIN, F. J. & NETTLETON, P. F. (2006) Genomic characterisation of a novel poxvirus contributing to the decline of the red squirrel (*Sciurus vulgaris*) in the UK. *Journal of General virology* **87,** 2115-2125

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

REYNOLDS, J. C. (1984) Details of the geographical replacement of the red squirrel (*Sciurus vulgaris*) by the grey squirrel (*Sciurus carolinensis*) in eastern England. *Journal of Animal Ecology* **54,** 149-162

RUSHTON, S. P., LURZ, P. W. W., GURNELL, J. & FULLER, R. (2000) Modelling the spatial dynamics of parapoxvirus disease in red and grey squirrels: a possible cause of the decline of the red squirrel in the UK. *Journal of Applied Ecology* **37,** 997-1012

RUSHTON, S. P., LURZ, P. W. W., GURNELL, J., NETTLETON, P., BRUEMMER, C., SHIRLEY, M. D. F. & SAINSBURY, A. W. (2006) Disease threats posed by alien species: the role of a poxvirus in the decline of the native red squirrel in Britain. *Epidemiology and Infection* **134,** 521-533

SAINSBURY, S. Q. & GURNELL, J. (1995) An investigation into the health and welfare of red squirrels, *Sciurus vulgaris*, involved in reintroduction studies. *Veterinary Record* **137,** 367-370

SAINSBURY, A. W., NETTLETON, P., GILRAY, J. & GURNELL, J. (2000) Grey squirrels have high seroprevalence to a parapoxvirus associated with deaths in red squirrels. *Animal Conservation* **3,** 229-233

SAINSBURY, A. W. & WARD, L. (1996) Parapoxvirus infections in red squirrels. *Veterinary Record* **138,** 400

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

SHORTEN, M. R. (1954) Squirrels. Collins

THOMAS, K., TOMPKINS, D. M., SAINSBURY, A. W., WOOD, A. R., DALZIEL, R., NETTLETON, P. F. & MCINNES, C. J. (2003) A novel poxvirus lethal to red squirrels (*Sciurus vulgaris*). *Journal of General Virology* **84,** 3337-3341

TOMPKINS, D. M., SAINSBURY, A. W., NETTLETON, P., BUXTON, D. & GURNELL, J. (2002) Parapoxvirus causes a deleterious disease in red squirrels associated with UK population declines. *Proceedings of the Royal Society B: Biological Sciences* **269,** 529-533

TOMPKINS. D. M., WHITE, A. R. & BOOTS, M. (2003) Ecological replacement of native red squirrels by invasive greys driven by disease. *Ecology Letters* **6,** 189-196

VIZOSO, A. D. (1968) A red squirrel disease. *Symposia of the Zoological Society of London* **24,** 29-38

First cases of squirrelpox in red squirrels (*Sciurus vulgaris*) in Scotland

C. J. McInnes, L. Coulter, M. P. Dagleish, et al.

Veterinary Record 2009 164: 528-531
doi: 10.1136/vr.164.17.528