

Role of small mammals in the persistence of Louping-ill virus: field survey and tick co-feeding studies

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Abstract. Louping-ill (LI) is a tick-borne viral disease of red grouse, *Lagopus lagopus scoticus* Lath. (Tetraonidae: Galliformes), and sheep, *Ovis aries* L. (Bovidae: Artiodactyla), that causes economic loss to upland farms and sporting estates. Unvaccinated sheep, grouse and mountain hares, *Lepus timidus* L. (Leporidae: Lagomorpha), are known to transmit LI virus, whereas red deer, *Cervus elaphus* L. (Cervidae: Artiodactyla), and rabbits, *Oryctolagus cuniculus* L. (Leporidae: Lagomorpha), do not. However, the role of small mammals is unknown. Here, we determine the role of small mammals, in particular field voles, *Microtus agrestis* L. (Muridae: Rodentia), in the persistence of LI virus on upland farms and sporting estates in Scotland, using field sampling and non-viraemic transmission trials. Small mammals were not abundant on the upland sites studied, few ticks were found per animal and none of the caught animals tested seropositive to LI virus. Laboratory trials provided no evidence that small mammals (field voles, bank voles, *Clethrionomys glareolus* L. (Muridae: Rodentia), and wood mice, *Apodemus sylvaticus* L. (Muridae: Rodentia), can transmit LI virus between cofeeding ticks and, in the field, LI virus was prevalent only in areas with known LI virus competent hosts (grouse, mountain hares or unvaccinated sheep) and absent elsewhere. In contrast to the case of tick-borne encephalitis (TBE) virus in Europe, it is concluded that small mammals seem to be relatively unimportant in LI virus persistence.

Key words. *Ixodes ricinus*, cofeeding, Louping-ill, non-viraemic transmission, small mammals, ticks, voles, Scotland.

Introduction

Tick-borne pathogens have a major impact on human, livestock and wildlife populations (e.g. Sonenshine & Mather, 1994). One of the main current concerns in epidemiology is how these pathogens persist, even with appropriate management. In this study we consider the Louping-ill (LI) virus (Flaviviridae), which is the only tick-borne virus in the U.K. known to cause disease in vertebrates (Reid, 1984). LI affects the central nervous system of both red grouse, *Lagopus lagopus scoticus* Lath., and sheep, *Ovis aries*

L., causing significant economic losses to agriculture and areas with commercially exploited wildlife. Up to 78% of infected red grouse may die of LI, whereas mortality in sheep is lower and varies considerably, depending on breed and virus prevalence (Reid, 1975; Reid *et al.*, 1978). LI virus is transmitted by the three-host sheep tick, *Ixodes ricinus* L. For detailed life history see Lees & Milne (1951). Larval and nymph stages of the sheep tick feed on a range of animals, whereas adults require larger mammal hosts and rarely feed on birds or small mammals. Although transstadial transmission of LI virus occurs, transovarial transmission has not been recorded in *I. ricinus* (Gaunt, 1997). Hence, larval ticks are not infected prior to feeding, and nymphs or adults are necessary to infect a host with the virus.

On British moors and upland farms only sheep and red grouse produce a sufficient viraemia to infect ticks (Reid &

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Doherty, 1971; Reid, 1975). Sheep can be effectively removed as hosts by vaccination against LI virus and the use of acaricides. Grouse are thought not to infect a large proportion of ticks because of their high mortality rate and rapid death when infected (Reid, 1984; Hudson & Dobson, 1991). Furthermore, because grouse are rarely hosts to adult ticks, they alone cannot sustain tick populations. However, despite good sheep management, LI virus persists, suggesting that alternative wildlife hosts maintain the disease (Hudson *et al.*, 1996).

Mammalian wildlife tick hosts common in upland Britain, namely red deer, *Cervus elaphus* L., mountain hares, *Lepus timidus* L., and rabbits, *Oryctolagus cuniculus* L., do not produce a sufficient viraemia to infect ticks (Beasley *et al.*, 1978; Reid, 1984). Of the common rodents, rats, *Rattus norvegicus* L., bank voles, *Clethrionomys glareolus* L., and wood mice, *Apodemus sylvaticus* L., produce low level viraemia, insufficient for virus transmission and only 8% of field voles, *Microtus agrestis* L., produce appreciable viraemia (Reid, 1984). However, experiments by Jones *et al.* (1997) have shown that, although mountain hares produce only a low level of viraemia, transmission of LI virus can occur between infected and uninfected ticks co-feeding simultaneously on the same individual mountain hare, i.e. non-viraemic (non-systemic) transmission. Co-feeding trials conducted on red deer and rabbits found no evidence of either viraemic or non-viraemic transmission. That study, along with mathematical modelling (Norman *et al.*, 1999), suggested that mountain hares can help LI virus persist. The role of other wildlife, such as small mammals, in the persistence of LI virus is not known.

LI virus is the western variant of the more extensive tick-borne fever (TBE) antigenic complex of viruses (de Zanolto *et al.*, 1996; Gould *et al.*, 1997). TBE virus causes clinical illness in humans in Europe, where small mammals, primarily yellow-necked mice, *Apodemus flavicollis* L., carry significant numbers of *I. ricinus* ticks and can transmit TBE virus through non-viraemic virus transmission between co-feeding ticks (Labuda *et al.*, 1993a, b). Thus, small mammals are considered instrumental in the persistence of TBE virus. Small mammals are also important reservoirs for many other pathogens and diseases throughout the world, such as *Leptospira* in Slovakia (Stanko *et al.*, 1996), toxocarasis (Dubinsky *et al.*, 1995), Lyme disease (Humair *et al.*, 1993; Vanbuskirk & Ostfeld, 1995), Rocky Mountain spotted fever (Kollars, 1996) and granulocytic ehrlichiosis in Minnesota (Walls *et al.*, 1997). Small mammals occur throughout Britain, the most frequently trapped species in the uplands being voles, *Microtus* spp., and shrews *Sorex* spp. (Redpath *et al.*, 1995; Redpath & Thirgood, 1997). If these species are important tick hosts and can transmit LI virus non-viraemically, they may be important in the persistence of LI virus, with adverse consequences for sheep and red grouse production.

In this study we aimed to determine the role of small mammals in the persistence of LI virus on upland sheep farms and moorland sporting estates in Scotland, using both field sampling and laboratory-based non-viraemic transmission studies.

Methods

Small mammal population index

Small mammal abundance was estimated and serological surveys conducted at 11 upland farms and estates in Scotland between July and September 1998. Farms and estates were located in Caithness (site C1), Sutherland (site S1), Morayshire (sites M1 and M2), Perthshire (site P1) and Argyll (sites A1 to A6), and ticks and LI were known to occur in all areas, according to local veterinary records. July to September was the period chosen for the surveys, to maximize vole numbers caught and to maximize tick numbers counted on each trapped mammal. Field vole numbers are usually lowest in spring and their numbers grow over the summer to a peak in autumn (Randolph, 1975; Redpath *et al.*, 1995). Tick questing studies were carried out by Laurenson *et al.* (unpublished data) using tick counts on mountain hares on study site M1 between 1993 and 1996. In contrast to the bimodal peaks in tick questing activity recorded in England (Lees & Milne, 1951; Ogdan *et al.*, 1997), Laurenson *et al.* found one seasonal peak for each tick stage. Larval and nymphal activity peaked between May and August, and adult activity peaked in August. Other studies conducted on upland sites with similar long cold winters and short summers have also found unimodal seasonal patterns in tick questing activity (e.g. Hendrick *et al.*, 1938; Steele & Randolph, 1985).

At each site, the relative abundance of small mammals was surveyed, under licence from Scottish Natural Heritage, using snap-trap transects (after Redpath & Thirgood, 1997, 1999). Transects of two lines, 2 m apart, each comprising 50 unbaited snap-traps were set over two consecutive nights to produce an abundance index of the number of animals caught per 100 trap-nights. In upland areas this is considered to be a more accurate and efficient method of estimating abundance than live trapping, mark-recapture or counting vole-sign (Redpath *et al.*, 1995). Two transects were set in each of the 11 sites, preferentially in areas of rough grassland to maximize the probability of catching field voles (see Redpath *et al.*, 1995). Trap lines were checked daily, tick numbers were counted on each trapped mammal and lung tissue was removed, frozen and later analysed for presence of LI antibody.

LI sero-prevalence

The sero-prevalence of LI virus at each site was analysed by taking serum samples from 10 to 32 six-month-old sheep (10 sites) or, where sheep were absent, from 90 recently shot red deer (one site). At the three sites where mountain hares were present, serum or lungs were collected from hares culled by gamekeepers. Sero-prevalence of LI virus in sheep was compared between vaccinated and unvaccinated flocks and between areas with and without other known LI virus-transmitting hosts, namely grouse or mountain hares.

Haemagglutination-inhibiting antibody (HAI) tests were undertaken on sera from sheep, red deer and mountain hares using chick red blood cells as described by Clark & Casals

Table 1. Abundance indices and tick numbers on small mammals caught at 11 sites in Scotland. *Abundance index is calculated as the number of small mammals caught per 100 trap nights (mean of 11 sites)

Species	No. caught	% of total	Abundance Index*		Ticks per mammal		
			Mean	SD	Mean	SD	Range
Field vole	61	75.3	1.7	2.3	1.4	3.0	0–20
Common shrew	11	13.6	0.3	0.7	0.4	0.7	0–1
Wood mouse	5	6.2	0.1	0.4	1.2	1.4	0–3
Bank vole	4	4.9	0.1	0.5	1.8	2.1	0–4
All species	81	100	2.3	2.4	1.3	2.6	0–20

(1958). Lung tissue from all small mammals caught as part of the population index estimate was also analysed for HAI. Reciprocal HAI titres of more than 20 HAI units were regarded as sero-positive.

Non-viraemic transmission

The isolate of LI virus used in this study was isolated in 1993 from an engorged female *I. ricinus* removed from a mountain hare in Inverness-shire, Scotland. The virus was initially isolated using pig stable (PS) kidney cell monolayers and subsequent virus stocks were derived by a single intracerebral passage in newborn mice. The nucleotide and deduced amino acid sequence of a portion of the envelope gene confirmed its identity as LI virus (unpublished data).

Virus assay. Individual ticks were homogenized in a microtissue grinder in 1 ml of EMEM containing 10% FBS and appropriate antibiotics to inhibit bacterial growth. Virus titration of small mammal blood and tick-derived material was performed in PS cells grown in EMEM supplemented with 3% FBS and incubated at 37 °C for 4 days, prior to fixation and staining. Virus isolated from individual ticks was confirmed to be LI virus by sequence analysis. Virus neutralization assays on hare sera were undertaken using PS cells and LI virus as previously described (Davies *et al.*, 1986).

Parenteral inoculation of ticks. A laboratory colony of uninfected *I. ricinus* was maintained as described by Jones *et al.* (1988). During the intervals between feeding, ticks were held in perforated tubes at 19 °C and 85% relative humidity. Cohorts of 30 laboratory-reared nymphs were fed to repletion on uninfected Syrian hamsters. Within a day of completing engorgement nymphs were inoculated intracoelomically, bypassing the gut, with approximately 1.0 µl LI virus (estimated 5.0 log₁₀ pfu/tick) using a drawn capillary tube. To confirm that this method successfully inoculated ticks with virus, a subsample of 10 moulted female ticks (42 days after engorgement as nymphs) was assayed individually for virus infectivity, and 100% infectivity was found. The injection method caused 20% mortality, but there was no subsequent virus-induced mortality of ticks.

Co-feeding of ticks on rodents. Non-viraemic transmission trials were conducted, under a Home Office licence, on 15 wood mice, 14 bank voles and 14 field voles obtained from field sites in Oxfordshire. Each was infested with four virus-

Table 2. Small mammal abundance indices and number of ticks counted per small mammal caught, by site. The abundance index is the number of small mammals caught per 100 trap nights. Of the 101 ticks counted, all were larvae except for two nymphs and one adult. Site key: M, Morayshire; C, Caithness; P, Perthshire; S, Sutherland; A, Argyll

Site	Abundance index	Ticks per mammal	
		Mean	Range
M2	2.1	1.4	0–3
C1	2.3	3.0	0–20
P1	0.5	0.3	0–1
M1	1.8	0	0
S1	5.7	1.8	0–8
A1	1.3	2.4	0–6
A2	2.2	0.5	0–1
A3	2.8	1.3	0–4
A4	2.7	0	0
A5	2.8	0.2	0–1
A6	3.3	0	0

infected adult *I. ricinus* (donors) in a retaining chamber. In the same retaining chamber, 10 uninfected nymphs (recipients) were added. Replete adults, 6–7 days post-attachment, were assayed for virus on the day of engorgement. Recipient nymphs were assayed for virus 12 days post-engorgement. On day 14 post-attachment of ticks, serum samples were taken from the rodents and antibody neutralization assays were undertaken using standard procedures (Davies *et al.*, 1986). The antibody titres given are the reciprocal of the dilutions, representing the 50% neutralization endpoints.

Results

Small mammal abundance, ticks and LI virus prevalence

From the 11 sites surveyed, the most commonly trapped animal was the field vole, comprising 75.3% of all captures. Common shrews, *Sorex araneus* L., comprised 13.6% of captures, whereas wood mice were trapped at only three sites and bank voles at one site (see Tables 1 and 2 for abundance indices). Abundance indices were relatively low, 2.3 ± 2.4

Table 3. Ticks on small mammals relative to mountain hares or red deer at the same sites during the same time period (July to end of September). Sites where sheep were the only other tick hosts were not used, because acaricides were applied to sheep. ++, ticks too numerous to count. Site key: M, Morayshire; C, Caithness; P, Perthshire

Site	Ticks/small mammal		Ticks counted per mountain hare or red deer*				
	<i>n</i>	Mean (\pm SD) Larvae	<i>n</i>	Mean (\pm SD) Larvae	Nymphs	Adults	Total ticks
M2	8	1.5 (\pm 1.3)	10	not recorded	8.3 (\pm 7.5)	3.4 (\pm 3.0)	11.7 (\pm 10.1)
C1	9	3.0 (\pm 6.5)	14	++	++	161.7 (\pm 108.7)*	161.7 (\pm 108.7)
P1	3	0.3 (\pm 0.6)	14	15.0 (\pm 19.7)	10.1 (\pm 6.8)	13.2 (\pm 10.6)	38.2 (\pm 32.2)
M1	10	0	12	16.8 (\pm 20.9)	10.1 (\pm 10.7)	4.5 (\pm 4.4)	31.3 (\pm 30.2)

(mean \pm SD) per 100 trap-nights for all species at all sites, even at the site where the most mammals were caught, where the index was 5.7 ± 5.2 per 100 trap-nights. Of the 81 individual mammals caught, none tested sero-positive to LI virus and they carried only 1.3 ± 2.6 ticks (Table 1). Of the 101 ticks counted on small mammals, 98 were larvae, whereas only two nymphs and one adult tick were found. No more than one tick stage was found on the same small mammal.

To gauge the importance of small mammals as tick hosts relative to other available mammal hosts, we compared tick numbers counted on small mammals with those on mountain hares and red deer during the same time period, July to September 1998, at the same sites (Table 3). These data indicate that the low tick numbers observed on small mammals were not due merely to a lack of questing ticks during this period.

Analysis of the prevalence of LI shows that virus was absent where sheep were vaccinated and grouse and hares are absent (Table 4). LI was present only where known LI virus transmitters are present. LI prevalence (arcsin-transformed) was significantly lower at sites where LI competent hosts (hares, grouse or unvaccinated sheep) were absent than at sites where they were present.

Transmission of LI virus between cofeeding ticks

Virus assays on the 172 donor female ticks revealed $1.7 \pm 1.9 \times 10^2$ (mean \pm SD) pfu/tick. None of the 346 recipient nymphs which co-fed with the virus-infected adults acquired virus (Table 5). Of the 43 rodents used in this study, none developed a detectable viraemia ($<2.0 \log_{10}$ pfu/ml of blood). However, 69.8% had a detectable antibody response to LI virus, showing that the majority of hosts had been exposed to virus, and neither viraemic nor non-viraemic transmission occurred.

Discussion

Five important findings suggest that small mammals are unimportant in the epidemiology of LI virus in Scotland. First,

Table 4. LI prevalences in 6-month-old sheep at all 11 sites in Scotland, in relation to known LI competent hosts (i.e. grouse, mountain hares, or unvaccinated sheep). At site C1 sheep were not present, and at site P1 the sheep were not serologically surveyed. Therefore, at these sites, the LI prevalence presented was from red deer and mountain hares, respectively. There was a significantly higher LI prevalence (arcsine-transformed) in the presence of LI competent hosts than in their absence (one-tailed Mann-Whitney *U*-test: $N=8, 3, W=7.5, P=0.018$). Site key: M, Morayshire; C, Caithness; P, Perthshire; S, Sutherland; A, Argyll.

Site	Hares present?	Grouse present?	Sheep vaccinated?	Louping-ill prevalence (%)	<i>n</i>
M2	Y	Y	Y	40.0	10
C1	N	Y	–	45.6	90
P1	Y	Y	Y	21.4	14
M1	Y	Y	Y	16.0	25
S1	N	Y	N	100	30
A1	N	N	N	0	32
A2	N	N	N	12.9	31
A3	N	N	N	3.3	30
A4	N	N	Y	0	20
A5	N	N	Y	0	15
A6	N	N	Y	0	27

small mammals were not abundant on the upland sites studied. Second, few ticks were found per animal. Third, none of the caught animals tested sero-positive to LI virus. Fourth, laboratory studies indicated that the three species of small mammal tested do not permit LI virus transmission between infected and uninfected ticks, even though the majority of individuals did sero-convert. Finally, LI virus in the field was present only where known transmitters (unvaccinated sheep, mountain hares or grouse) occurred, and was absent in their absence.

Small mammals were not abundant at any of the sites, for example we caught 1.7 field voles per 100 trap nights in rough grassland, compared with 12.7 (mean of four sites) caught by Redpath *et al.* (1995) in similar habitat in the Southern Uplands of Scotland. Population cycles occur in some vole populations, so it is possible that they were at the trough in a cycle at the time of sampling. However, recent studies have shown that

Table 5. Results of non-viraemic transmission trials using co-feeding ticks on bank voles, field voles and wood mice, showing virus infectivity of donor adult ticks, the resulting number of recipient nymphs that became infected, and the proportion of rodents sero-converting. Those rodents said to sero-convert had reciprocal antibodies of between 1/4 and 1/32, whereas those recorded as not sero-converting were less than 1/4.

Species	<i>n</i>	No. sero-positive	% sero-positive	Donor pfu/tick × 10 ³ (± SD)	No. recipients infected/no. tested
Bank vole	14	9	64.3	1.82 (± 2.13)	0/112
Field vole	14	10	71.4	1.61 (± 1.75)	0/102
Wood mouse	15	11	73.3	1.59 (± 1.90)	0/132
Total	43	30	69.8	1.67 (± 1.93)	0/346

travelling waves in vole cycles cause asynchrony between populations (Ranta & Kaitala, 1997; Lambin *et al.*, 1998), so it may be unlikely that all sites would be at a population trough simultaneously, especially as sites were so widespread.

Very few ticks (mean of 1.3) were found on trapped small mammals, although this is likely to be an underestimate because some ticks detach after host death. If our field study occurred when tick questing was between peaks of activity, we would expect other hosts, such as red deer and mountain hares, also to carry few ticks. However, the fact that deer and hares carried large tick burdens suggests that ticks were abundant during the study months and that small mammals are relatively insignificant in maintaining the tick population. Almost all ticks counted on small mammals were larvae and, because unengorged larvae are uninfected, there may be little scope for LI virus transmission in the wild even though 8% field voles reacted viraemically to LI virus infection in laboratory tests by Reid (1984). It is notable that the laboratory study showed that 70% of the 43 small mammals tested sero-converted when exposed to LI virus, but none of the 81 trapped wild mammals had sero-converted. This confirms that, in the field, none of the trapped mammals had been exposed to LI virus. Furthermore, for non-viraemic transmission to occur, an infected nymph or adult tick must be co-feeding alongside susceptible larvae or nymphs. This requires that the two tick stages quest at the same time. Although the seasonal peaks of questing larvae and nymphs did coincide (Laurenson *et al.*, unpublished data, at study site M1), none of the 81 small mammals caught in our field study had more than one tick stage feeding on any part of their bodies.

Larger sample sizes of field-sampled small mammals, by sampling in a further season and at sites with higher small mammal populations, may have revealed some cases that were sero-positive to LI virus, or that had nymphs and larvae co-feeding. The proportions of these, however, would still be small and, more importantly, our laboratory-based studies found that neither viraemic nor non-viraemic transmission occurred between ticks co-feeding on field voles, bank voles or wood mice. This indicates that these species are unlikely to be important hosts for the persistence of LI virus. Field sampling found shrews to be the second most abundant small mammal at our study sites, comprising 14% of all catches, so non-viraemic transmission trials need to be conducted on shrews to positively confirm that they have no role to play in LI virus persistence. Apart from small mammals, other alternative tick hosts require investigation, such as roe deer, *Capreolus*

capreolus L., predators, such as foxes, *Vulpes vulpes* L., and stoats, *Mustela erminea* L., and upland breeding birds, such as curlew, *Numenius arquata* L., snipe, *Gallinago gallinago* L., or meadow pipits, *Anthus pratensis* L. Our serological survey, however, suggests that alternative hosts are not important; LI virus was only prevalent in areas with known LI virus transmitters, such as grouse, mountain hares or unvaccinated sheep. LI virus was absent in the absence of these hosts, suggesting that other tick hosts are relatively unimportant in the epidemiology of LI virus.

The role of small mammals in the epidemiology of LI virus and ticks in upland Britain contrasts strongly with the role of small mammals in the persistence of TBE virus and ticks in central Europe. LI virus is part of the TBE antigenic complex of viruses and has diverged relatively recently, being confined to the U.K. and Ireland, and parts of Norway and France (de Zotto *et al.*, 1996; Gould *et al.*, 1997). In central Europe, yellow-necked mice, that inhabit woodland habitats primarily, are considered instrumental in the persistence of TBE virus because they carry significant numbers of *I. ricinus* ticks and permit efficient non-viraemic transmission of TBE virus (Labuda *et al.*, 1993a, b). This fundamental difference in epidemiology between LI and TBE viruses may be due to both the difference in habitat (upland moors vs. woodland), and a difference in the hosts (mice vs. sheep) which shaped the evolution of these viruses.

To conclude, we found no evidence that small mammals are important in the epidemiology of the LI virus in upland Scotland. Co-feeding trials need to be conducted on shrews to confirm that they play no role in LI virus persistence. However, it appears unlikely that they are important because of the low probability of coincidental co-feeding of nymphs and larvae due to their low tick burdens. Small mammals may help a small proportion of larval ticks survive, depending on their density relative to alternative tick hosts. Although a small proportion (8%) of field voles may have the theoretical capability to transmit LI virus viraemically (Reid, 1984), it is likely to be uncommon in the wild because 98% of ticks feeding on voles were uninfected larvae.

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